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Endocrine and Follicular Dynamics during the Perideviation Period in Cattle

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ENDOCRINE AND FOLLICULAR DYNAMICS DURING THE PERIDEVIATION PERIOD IN CATTLE

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by
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ABSTRACT

Ovarian hyperstimulation used in conjunction with embryo transfer techniques is an assisted reproductive technology that aids cattle producers in reaching their reproductive, genetic, and financial goals; however, viable embryo recovery results are inconsistent, possibly because the reproductive endocrine profile is poorly understood. The objective of these experiments was to evaluate the endocrinological and ovarian dynamics surrounding follicular deviation in hyperstimulated cows.

In Experiment 1, the perideviation period (PDP; largest follicle diameter from 8 - 10- mm) was examined in Hyperstimulated (50 mg FSH) or Control (saline) dry Holstein cows (n = 4 / group). Daily blood samples began at the initiation of an engineered follicular wave and ended at ovulation. Jugular blood samples were collected every 15 min during the PDP. Trans-rectal ultrasonography was performed at 8 hr intervals prior to and 4 hr intervals during the PDP, and daily thereafter until ovulation. Serum samples were analyzed for LH and FSH concentrations. Results indicated that Controls experienced a longer PDP (P = 0.003) and more LH pulses in the PDP (P = 0.008) without increasing the overall concentration or the number of LH pulses per hour. Hyperstimulated cows tended to have more follicles > 7- mm at the initiation (P = 0.059) and the cessation (P = 0.054) of the PDP, experienced a faster growth rate (P = 0.018), and more ovulations (P = 0.087).

In Experiment 2, the PDP (6 – 10- mm) was evaluated in hyperstimulated beef cattle with 1 follicle ≥ 6 mm (Single – Follicle Group; n = 8) or all follicles ≥ 6 mm (Multi – Follicle Group; n = 7). Trans-rectal ultrasonography and blood collection were

conducted to evaluate follicular dynamics and FSH and E2 concentrations. In the Single – Follicle Group, non-retained follicles were ablated upon reaching 6- mm (approximately 3.5 ablations per cow). When the largest follicle reached 10- mm in the Single – Follicle Group, that follicle was aspirated and the follicular fluid stored for future assay. When the largest follicle reached 10- mm in the Multi – Follicle Group, fluid from the 10 largest follicles (≥ 8 mm) was collected, pooled, and stored for future assay. The results of this study indicated that the presence of more than one follicle ≥ 6 mm in a hyperstimulated follicular wave gradually increased serum E2 and suppressed FSH when the largest follicle was detected to be 10- mm. Prior to the cessation of the PDP, the Multi – Follicle Group experienced a gradual increase in serum E2 and marked decrease in FSH over the Single – Follicle Group. Additionally, there was a trend towards increased total follicular fluid E2 in the Multi – Follicle Group ($P = 0.076$); however, there was not a direct relationship between the number of large follicles and E2 production. This may be indicative of a compensatory mechanism to produce a threshold amount of systemic E2 perhaps necessary to regulate FSH or that E2 production potential was inhibited by the sub – luteal Progesterone concentrations in the CIDR inserts used in this experiment.

In addition to providing a better understanding of the endocrine and follicular dynamics of hyperstimulation, these results indicated; 1) an apparent enhanced competition for dominance among follicles in hyperstimulated cows, 2) the largest follicles in a hyperstimulated follicular wave require less time to reach 10- mm and there was a tendency towards enhance ovulation rates 3) endogenous LH was sufficient to support one or multiple dominant follicles, and 4) endocrine deviation and follicular E2

production may be postponed in a hyperstimulated follicular wave. Future research is needed to understand the roles of individual follicles of various diameters and the impact of E2 production (in the face of varying Progesterone concentrations) on the hyperstimulatory response, capacity for oocyte fertilization, and embryo production.

DEDICATION

I would like to dedicate this thesis to my family. My parents for their unyielding love, support, and advice, and my siblings, Jay and Dana, for never letting me forget that there are more important things in life than me. Without all of you, I never would have tried.

ACKNOWLEDGMENTS

First I would like to recognize Dr. John Gibbons for his guidance, patience, and commitment. The around-the-clock animal trials were made possible because of him. I would also like to thank Dr. H. Lee Higdon and Dr. Scott Pratt. Their support and encouragement helped make this experience worthwhile.

The Clemson University LaMaster Dairy and Simpson Experiment Station did an excellent job providing research animals and helping me handle sample collection. Thank you for your time and assistance. I would like to thank Nancy Korn for helping me find a way to handle the endless samples and the undergraduates who worked night and day to make these research projects possible. Dr. Sandra Gray and Jane Owenby, thank you for your wonderful guidance, insight, and assistance. I am eternally grateful to my lab mates, Erin Curry, Jillian Fain, Colette Floyd, and Kristine Vernon who were there for me when I needed them most and to all the other graduate students who made work enjoyable.

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CHAPTER ONE

INTRODUCTION

The maximum reproductive efficiency of a cow is approximately 1 calf per year; however, a bull is capable of spreading his genetics over many cows and quickly manipulating cattle genetics. Ovarian hyperstimulation, in conjunction with other assisted reproductive technologies, is commonly used in the cattle industry to increase the rate of maternal genetic contributions. The genetics of superior females are now able to proliferate within surrogate dams and embryos can be frozen for transport around the globe and through time. The American Embryo Transfer Association (AETA) monitors embryo collections across the nation and their 2006 data are summarized in Table 1.1.

Unfortunately, there is tremendous variability in the ovarian hyperstimulatory response among cattle and from cycle to cycle. By better understanding the endocrine dynamics associated with the ovarian hyperstimulatory response, the cattle industry can begin to manage cattle to produce a more uniform and optimal response to superovulation protocols. Progress has been made to better understand cattle during the periovulatory period; however, the perideviation period has rarely been addressed.

When the perideviation period was investigated, the experimental design rarely integrated an engineered follicular wave with controlled Progesterone. These two factors were of importance because research investigating the influence of Progesterone on gonadotropin profiles showed that Progesterone had the capacity to inhibit LH (Ginther *et al*, 2001). Also, engineered follicular waves are used in the cattle industry and may be associated with altered follicular dynamics. This seems reasonable since trans-vaginal

follicular ablations physically alter the ovaries, induce local inflammation, and subsequently increase ovarian blood flow.

The objectives of this research were first to correlate reproductive endocrine profiles with ovarian dynamics in control and hyperstimulated cows during the perideviation period (PDP). The second objective was to determine if the number of dominant follicles an animal was allowed to retain influenced the reproductive endocrine profiles before and during the PDP. The variable influence of endogenous Progesterone was perhaps muted in this study as a sub-luteal concentration of Progesterone was supplied to cows in both experiments.

Table 1.1. Embryo Collections for 2006 in the United States

Hyperstimulated and Flushed Dairy Donors	16,570
Total Viable Embryos Collected - Dairy	90,387
Average Number of Viable Embryos per Cycle - Dairy	5.46
Hyperstimulated and Flushed Beef Donors	35,323
Total Viable Embryos Collected - Beef	229,316
Average Number of Viable Embryos per Cycle - Beef	6.50
Total Hyperstimulated and Flushed Donors	**51,893**
Total Viable Embryos Collected	**319,703**

CHAPTER TWO

LITERATURE REVIEW

Estrous Cycle in Cattle

The Estrous cycle is defined as the period of time from one estrus to the next. In cattle, the ovarian tissue left behind from ovulation re-organizes to form a Progesterone producing corpus luteum (CL). The CL takes approximately 4 – 5 days from ovulation to gain Progesterone producing capacity in excess of 1 ng / ml and gain Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptors. The CL reaches in its maximal Progesterone producing capacity by Day 10 post - estrus (Hansel *et al,* 1973). The CL is made up of small luteal cells which express receptors for Luteinizing Hormone (LH) and contribute to Progesterone production in increasing concentrations when stimulated by LH and large luteal cells which produce most of the Progesterone and express most of the receptors for Prostaglandin E_2 and Prostaglandin $F_{2\alpha}$. Luteal regression is induced by uterine release of $PGF_{2\alpha}$ stimulated by the absence of a viable embryo (Niswender, *et al,* 2000). Luteal regression can also be induced by administration of $PGF_{2\alpha}$ (Youngquist and Threlfall, 2007).

Follicle Stimulating Hormone (FSH) and luteinizing hormone (LH) are dimeric glycoproteins produced in the gonadotroph cells of the pituitary gland's pars distalis region. The alpha subunit of these proteins is highly conserved among glycoproteins while the beta subunit is responsible for specificity and biological activity (Li and Ford, 1998). The primary role of FSH is to stimulate follicular growth (Adams *et al,* 1992)

while LH works to facilitate the growth of dominant follicles specifically (Ginther *et al,* 1998) and induce the ovulatory cascade (Chenault *et al,* 1975).

Receptors for FSH are found on the granulosa and theca interna cells of follicles (Xu *et al,* 1995). Surges of FSH recruit cohorts of follicles to grow and later compete for dominance in the face of decreasing concentration of FSH (Adams *et al,* 1992). Emergence, or the last ultrasound scanning session where the future dominant follicle appears at 4- mm, occurs concomitant with the peak of the FSH surge (Ginther *et al,* 1996). The future dominant follicle and its closest competitor begin to grow at different growth rates at deviation. In cattle, follicles are approximately 8.5- mm, express LH receptors on granulosa cells, and are estrogenic at deviation. The time period from the emergence of the future dominant follicle until it undergoes deviation is termed selection and lasts approximately 2.8 days (Ginther *et al,* 1996).

After a follicle undergoes deviation, it is considered dominant. Dominant follicles continue to grow in the face of low levels of FSH and instead respond to the tonic release of LH. Ovulatory and non-ovulatory dominant follicles produce Estradiol 17-β (E2; Martin *et al,* 1991) in increasing amounts as the follicle grows after deviation (Beg *et al,* 2002). Suppression of FSH is assisted by E2 (Ginther *et al,* 1996) and recruitment of follicles is postponed until the dominant follicle either ovulates or becomes atretic.

Luteinizing hormone is released in a tonic pattern through most of the estrous cycle, until the pre-ovulatory surge in LH that stimulates ovulation. Early in the cycle, LH is released in frequent pulses with low amplitude. As Progesterone production by the CL reaches its maximum (> 5 ng / ml), LH pulses grow in amplitude but their frequency

declines (Rahe *et al,* 1980). Progesterone can inhibit the average concentration of LH when present concentrations ≥ 50 mg and can have a negative impact on follicular growth when concentrations are ≥ 100 mg (Ginther *et al,* 2001). The pre-ovulatory surge of LH is triggered by increasing concentrations of E2 and decreasing concentrations of Progesterone (Youngquist and Threlfall, 2007).

Surges of FSH result in wave - like patterns of follicular growth termed follicular waves. Estrous cycles typically contain two follicular waves in cows and three or four follicular waves in heifers (Lucy *et al,* 1992). The first follicular wave in an estrous cycle is initiated by the secondary rise in FSH that accompanies the pre-ovulatory LH surge that stimulates ovulation of the follicle produced in the previous cycle (Youngquist and Threlfall, 2007). Rises in FSH precede follicular emergence by 1-2 days (Adams *et al,* 1992).

The end of an estrous cycle is marked by estrus, or sexual receptivity, which is commonly followed by ovulation. Progesterone levels decline as $PGF_{2\alpha}$ induces lyses of the CL. Concurrently, E2 production from the dominant follicle increases as the follicle grows without inhibition from Progesterone. As Progesterone declines, basal levels of LH increase and pulse frequency increases to approximately 1 pulse per hour (Hansel and Convey, 1983). The dominant follicle produces a threshold concentration of E2 that acts on the pituitary gland and results in a surge of LH. The onset of estrus typically coincides with the beginning of the pre-ovulatory surge of LH and lasts for an average of 12 – 18 hrs. Ovulation, stimulated by the LH surge, occurs 24 -30 hrs after the initiation of the

pre – ovulatory LH surge and marks the end of an estrous cycle (Youngquist and Threlfall, 2007).

Hypothalamic – Pituitary - Ovarian Axis

The bi-lobed pituitary gland is tightly regulated by the hypothalamus. Neurons from the hypothalamus travel into the posterior lobe of the pituitary termed the neurohypophysis, which originates from neural tissue. Most of the neurosecretory cells have their cell bodies in the hypothalamus, so neurohypophyseal products (oxytocin and anti – diuretic hormone for example) are synthesized in the hypothalamus. The adenohypophysis is the anterior lobe of the pituitaryand is derived from the oral cavity epithelium. It receives signals from the hypothalamus through the capillary plexus found in the median eminence termed the Hypothalamic – Hypophyseal Portal System (Dyce *et al,* 2002). For reproductive endocrinological regulation, Gonadotropin Releasing Hormone (GnRH), is produced by neurosecretory cells in the hypothalamic nuclei, enters the Hypothalamic – Hypophyseal Portal System through capillary fenestrations, and moves into the adenohypophysis to regulate gonodotropin productions and release. The gonadotropins enter the capillaries within the adenohypophysis which drain into the cavernous sinus (Dyce *et al,* 2002).

In cattle, GnRH is a decapeptide that travels from the hypothalamus to the adenohypophysis and is released in a pulse pattern (Ginther 1992). During the luteal phase of the estrous cycle, GnRH pulses are directly influence gonadotropin release and yield a pulse profile for LH (1 GnRH pulse ≈ 1 LH pulse) and a wave profile for FSH

release. The pre – ovulatory surge in LH and secondary rise in FSH is signaled by increased GnRH pulse frequency, stimulated by threshold concentrations of Estradiol 17-β reached in the presence of low Progesterone. The GnRH pulse frequency increases and stimulates the adenohypophysis to release increasing concentrations of LH and FSH (Youngquist and Threlfall, 2007).

The ovaries provide a feedback system for the hypothalamus and adenohypophysis. Growing follicles express FSH receptors and up-regulate LH receptor expression once they become dominant (Xu *et al,* 1995). Dominant follicles grow and produce E2 in response to low FSH concentrations and tonic LH (Ginther *et al,* 1996). The E2 production is limited by luteal Progesterone; however, once lysis of the CL occurs, the dominant follicle produces enough E2 to stimulate and increase GnRH pulse frequency and the subsequent pre-ovulatory surge in LH and FSH (Youngquist and Threlfall, 2007).

Ovarian Hyperstimulation in Cattle

The goal of ovarian hyperstimulation in cattle is to increase the number of dominant follicles capable of ovulation. More ovulations aims to result in an increase in the number of embryos produced after insemination and are later available for embryo collection (Hasler, 2003). The number of follicles that undergo deviation may be increased by manipulating the FSH surge with exogenous of FSH. By increasing the height and breadth of the FSH surge, more follicles are recruited. The continued exposure to FSH, despite endogenous decline, stimulates additional follicles to gain dominance.

This exploitation of the FSH surge and follicular wave enables an increase in dominant follicles (Gibbons *et al,* 1997).

The tonic release of LH may be largely responsibly for the development and maturation of large follicles. The administration of FSH has been shown to decrease the pulse pattern of LH in Freisian – Holstein heifers during the follicular phase (Ben Jebara *et al,* 1994). In this experiment, FSH administration began on Day 10 of the estrous cycle and luteolysis was induced on Day 12. The LH pulse pattern was assessed 6 – 14 hrs after the induction of luteolysis. The results of this study indicated that exogenous FSH decreased the pulse pattern of LH, decreased the average concentrations of circulating LH, increased serum E2, and increased ovulation rates (Ben Jebara *et al,* 1994). The administration of FSH has been shown to cause a decrease in the pulse patterned release of LH and a decrease in mean LH levels during Days 9-11 in heifers. The time points assessed were 8 - 20 hrs and 32 - 44 hrs after the initiation of FSH treatment. Estradiol was elevated in treatment animals' 8 – 20 hrs after the initiation of treatment and Progesterone was also higher in treatment animals. The results did not indicate that the decrease in LH pulses was due to negative feedback mechanisms from E2 or Progesterone (Gosselin *et al,* 2000). The modification in the release of LH may play a role in the variability of a large follicle's ability to respond to the LH surge and ovulate. While ovarian hyperstimulation has been shown to induce changes in the endocrine profiles early- and mid- cycle, it seems to have little effect on mean basal LH or the pulse frequency during the periovulatory period (Yadav *et al,* 1986).

In 1995, Price used ovariectomized Holstein heifers to investigate the direct relationship between FSH administration and suppressed LH pulse frequency. Heifers were ovariectomized 20 days before starting treatments with either 6 injections of FSH totaling 50 mg (n = 6) or saline (n = 5) injections 12 hrs apart. Blood samples were taken every 10 min for 8 hrs starting 12 hrs before initiation of treatment and again on Day 4 of the experiment. Results from this study showed no difference in LH pulse frequency in animals treated with FSH versus controls. The suppressive result of FSH administration on LH pulse patterens in intact females therefore may be mediated by the ovaries (Price, 1995).

Kemper Green *et al* (1996) investigated the relationship between FSH administration and steroid hormone profiles during the treatment period in Angus heifers. Heifer received twice - daily injections of either FSH or saline for up to 4 days beginning on Day 10 after a synchronized estrus. Follicular dynamics were monitored every 12 hrs via ultrasonography and blood samples obtained every 12 hrs for the first 48 hrs and then every 6 hrs for the remainder of the experiment. Ovaries were collected at 24, 48, 72, and 96 hrs after initiation of FSH injection in treatment animals and 24 and 96 hrs in controls. Follicular dynamics were recorded before follicular fluid was collected and blood was sampled from each animal for steroid hormone assays. Results from this study indicated an increase in plasma E2 within 36 hrs of the initiation of treatment. Additionally, there was an increase in the number of medium follicles (5 – 10 mm) by 60 hrs after treatment began. The follicular fluid increased in volume with treatment and the primary steroid

was E2. There was a temporal association between the follicular fluid E2 and serum E2 (Kemper Green *et al,* 1996).

CHAPTER THREE

ENDOCRINE DYNAMICS SURRONDING THE DOMINANT FOLLICLE SELECTION FROM 8 - 10- mm IN CATTLE

Objective

Cattle hyperstimulated with exogenous Follicle Stimulating Hormone (FSH) may experience changes in their endocrine profiles and ovarian dynamics during the perideviation period (PDP). These changes may be linked to the variable superovulatory responses and embryo production in the bovine embryo transfer industry. The objective of this study was to evaluate the endocrine profile (LH and FSH) and associated ovarian follicular development profiles during the PDP in cattle hyperstimulated with exogenous FSH.

Perideviation Period (PDP)

The measured period of time from the first ultrasonographic detection of an 8-mm follicle until the first detection a 10- mm follicle in an engineered follicular wave.

Hypothesis One

The release profile of LH and FSH will be suppressed in Hyperstimulated cows versus Control cows during the PDP.

Hypothesis Two

The number of large follicles (> 7- mm) will be enhanced in Hyperstimulated cows versus Control cows during the PDP.

Materials and Methods

Estrous synchronization

Estrus in mature, open, dry Holstein cows was synchronized using a 25 mg intramuscular (IM) injection of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ - ProstaMate®; Phoenix Scientific, Inc., St. Joseph, MO) to treat cows with corpra lutea (CL) at least 10- mm in diameter. Six to 11 days post estrus (Day 0) cows were treated with an additional IM injection of $PGF_{2\alpha}$, all follicles greater than 4- mm were destroyed via ultrasound-guided trans - vaginal ablation with a 5 - MHz transducer, and received a CIDR device (Eazi-breed CIDR; DEC International, NZ). Daily blood samples were collected until the start of the PDP. On Day 1, cows received a second $PGF_{2\alpha}$ IM injection to ensure luteal regression and indwelling jugular catheters were inserted and cows tethered to reduce catheter disruption. Free choice hay and water and dry – cow ration (once per day) were provided. After the PDP, CIDRs were removed and cows were scanned daily to monitor follicular development and again approximately 1 week later to determine ovulation rates.

Treatment Protocol

Forty eight hrs after ablation (concomitant with the emergence of a new follicular wave; Berfelt *et al*, 1994), cows were randomly divided into two groups (n = 4). The

Hyperstimulated group received a total of 50 mg FSH (Sioux Biomedical, Sioux Center, IA, Lot # 3097; 2.5 mg/ml) in 8 twice daily decreasing IM injections (3.5, 3.0, 3.0, 2.5, 2.5, 2.0, 2.0, 1.5 ml / injection). The Control group received IM saline injections of equal volume. Trans-rectal ultrasonography with a 7.5 MHz transducer was performed every 8 hrs to monitor follicular growth until an 8- mm follicle was detected. Blood samples were collected at 15-min intervals and follicular dynamics were mapped via ultrasonography every 4 hrs throughout the PDP

Whole Blood Processing

After collection, blood samples were allowed to clot at 20^{o}C for 12 – 24 hrs in 10 ml borosilicate glass tubes with plastic snap caps. Tubes were centrifuged for 20 – 30 min at 12,000 x g for optimal serum separation. After separation, serum samples were stored in plastic scintillation vials (7 ml, Fisher Scientific, Norcross, GA) at -20^{o}C in a non-frost free freezer until assayed.

LH Radioimmunoassay

Serum concentrations of LH at 15- min sampling intervals were measured in duplicate using the Clemson University Endocrine Physiology Lab standard ^{125}I RIA protocol for the detection of bovine LH (Appendix A). This RIA is a 3 day, double antibody, competitive binding assay with a range of detectability from 0.39 – 50 ng/ml. The first antibody was Bovine LH Kiser Antibody and the second antibody was Goat Anti-Rabbit IgG (Pel Freez Biologicals, Rogers AR Lot # 25815). LH concentrations were accepted if % Coefficient of Variations (%CV) were ≤ 15% for each set of samples.

Samples with %CV greater than 15% were re-assayed and duplicates greater than 2 standard deviations from the mean were discarded.

FSH Radioimmunoassay

Serum concentrations of FSH at hourly sampling intervals were measured in duplicate using a double antibody ^{125} I, 2 day competitive binding RIA procedure. The range of detectability for the FSH RIA was 0.31 – 20 ng/ml. The procedure (Appendix B) was based on the work of Dr. Parlow at the UCLA Medical Center's National Hormone and Peptide Program (Torrence, CA) and modified by Dr. S. Gray at the Clemson University Endocrine Physiology Lab (Clemson, SC). The NHPP supplied the bovine FSH for iodination and standards (AFP – 5332B), the first antibody (anti – oFSH – 1 A.S., RIA AFPC – 5288113Rb), and the Goat Anti – Rabbit IgG 2nd Antibody (Rockland Anti – Rabbit IgG Lot # 16528). FSH concentrations were accepted if %CV were ≤15% for each set of samples. Any samples with % CV greater than 15% were re-assayed and duplicates greater than 2 standard deviations from the mean were discarded. See above

Data Analysis

A Student's t-test was used to detect differences in serum LH and FSH concentrations and follicular dynamics. SAS 9.1 ANOVA was used to evaluate Group, Time, and Group by Time interactions. Fisher's Least Significant Difference Test was used to assess differences at specific times.

Assay Efficiencies

LH and FSH RIAs were performed on multiple assays. The intra-assay coefficients of variation for the LH assays ranged from 5.5 – 6.2% with an inter-assay

coefficient of variation of 5.8%. The intra-assay coefficients of variation for FSH were 8.7% and 10.3% resulting in an inter-assay coefficient of variation of 9.5%.

Results

Data analysis indicated that there were no differences between groups for the number of LH pulses per hour or average FSH concentration during the PDP. Data are presented as mean ± standard error. A pulse of LH was defined as two consecutive assay values in the Top 25 th percentile of all ordered values for an individual. The total LH pulses during the PDP were 6.8 ± 0.9 in the Control and 3.3 ± 0.3 in the Hyperstimulated group (P = 0.008). A summary of the PDP dynamics for Experiment 1 are in Table 3.1.

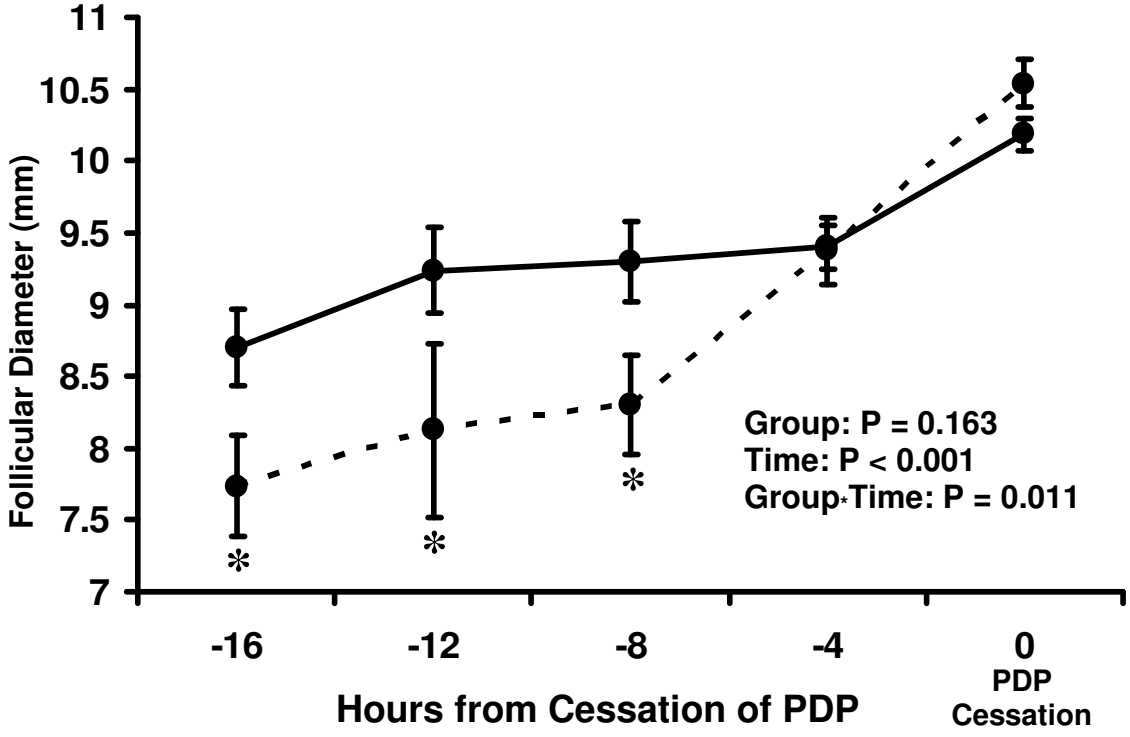
Table 3.1 Comparison of PDP Length, Growth Rate of the Largest Follicle, Average LH and FSH Concentrations, LH Pulse Frequency, and Total Ovulations in HS or Control Holstein cows (Experiment 1)

End point	Control Group	Hyperstimulated Group	P-value
PDP (hrs)	27.81 ± 2.05	15.38 ± 1.55	0.003
Growth Rate of Lgst Follicle (mm / hr)	0.07 ± 0.01	0.13 ± 0.01	0.018
Avg. LH Concentration (ng / ml)	0.86 ± 0.07	1.06 ± 0.08	0.111
LH pulses / hour	0.24 ± 0.01	0.22 ± 0.04	0.671
Avg. FSH Concentration (ng / ml)	0.79 ± 0.06	1.06 ± 0.10	0.066
Total Ovulations	1.00 ± 0.00	7.50 ± 3.18	0.087

Data are presented as mean ± SEM

The largest follicle from the Hyperstimulated group was compared with the largest follicle from the Control group in Figure 3.1. Data were normalized to the cessation of the PDP and incorporate data points from the last 16 hrs of the PDP. Data from individual cows normalized to the PDP cessation to compensate for the variability of follicular growth over time since the PDP was defined by physiological marker instead of time points.

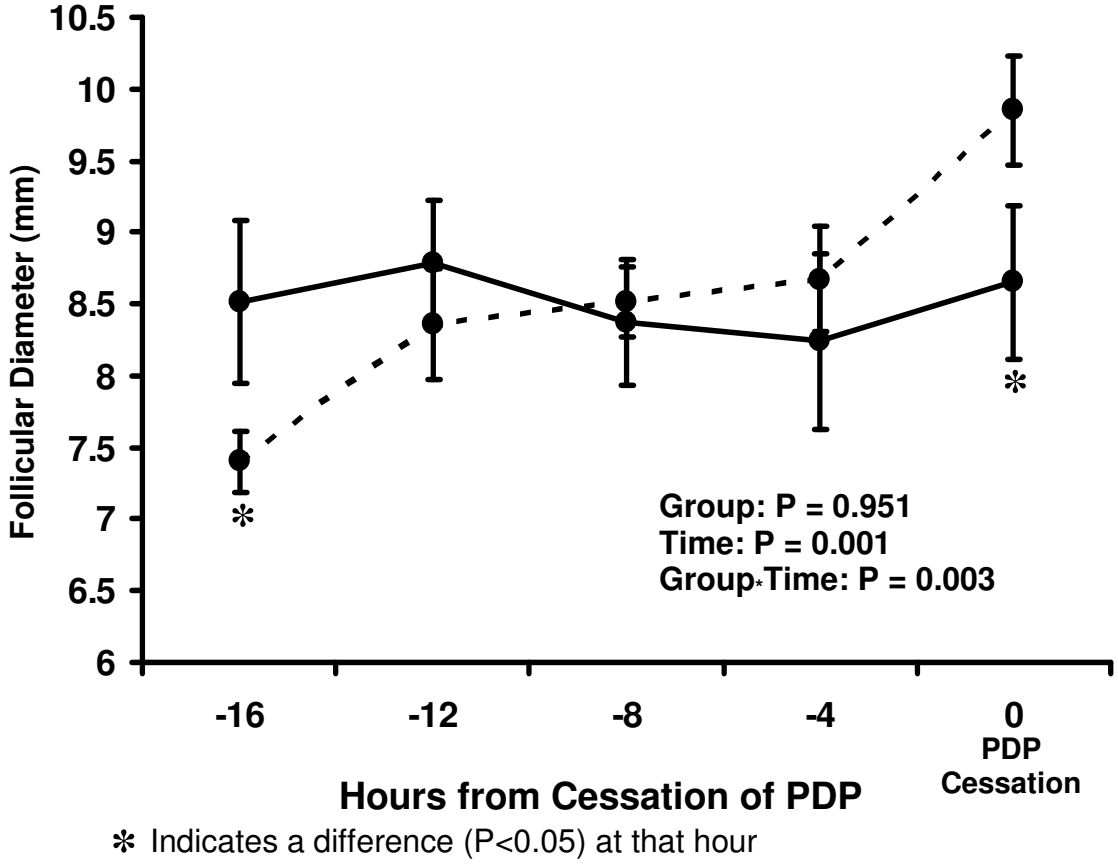
Figure 3.1. Follicular Diameter of the Largest Follicle Normalized to the Cessation of the PDP in Hyperstimulated (- -) and Control Holstein cows (—) in Experiment 1



* Indicates a difference (P<0.05) at that hour

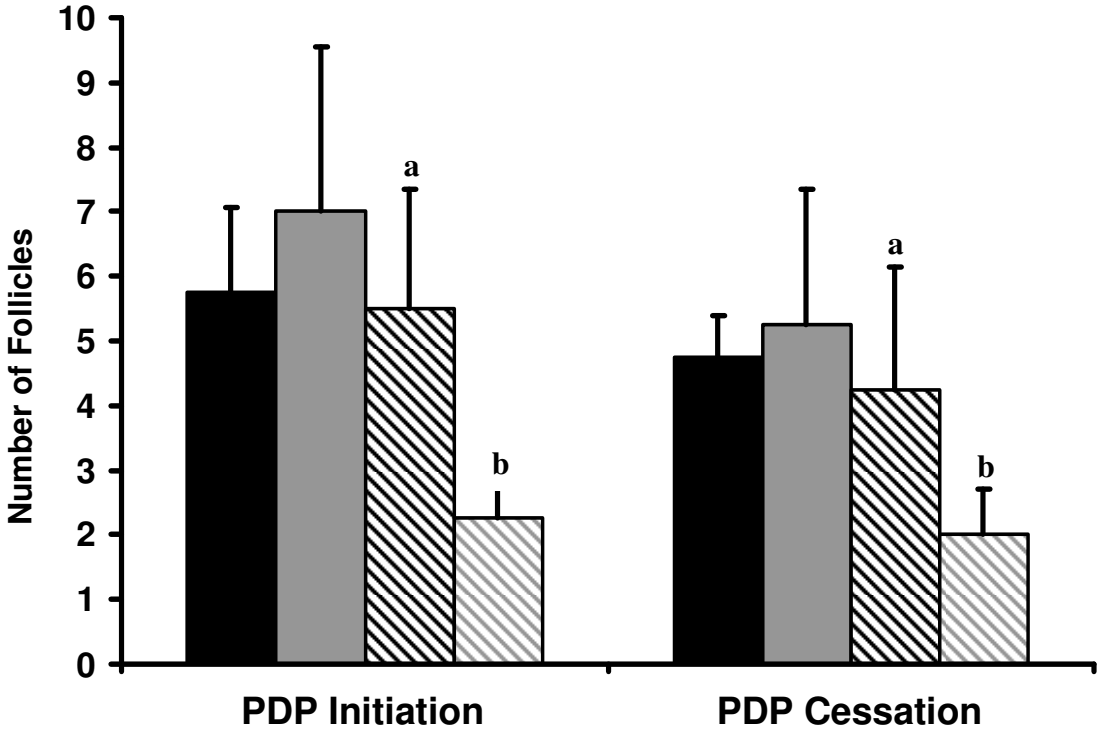
The second largest follicle from the Hyperstimulated group was compared with the second largest follicle from the Control group in Figure 3.2. Data are normalized to the cessation of the PDP and incorporate data points from the last 16 hrs of the PDP. Data from individual cows normalized to the PDP cessation to compensate for the variability of follicular growth over time since the PDP was defined by physiological marker instead of time points.

Figure 3.2. Follicular Diameter of the Second Largest Follicle Normalized to the Cessation of the PDP in Hyperstimulated (- -) and Control Holstein Cows (—) in Experiment 1



The number of follicles from 4 – 7- mm and the number of follicles > 7- mm were analyzed at the initiation and again at the cessation of the PDP in the Hyperstimulated Group versus the Control Group (Figure 3.3). There were more follicles > 7- mm in the Hyperstimulated Group at both the initiation and cessation of the PDP.

Figure 3.3. The Number of 4-7- mm (solid) and > 7-mm (striped) Follicles in Hyperstimulated (black) or Control (gray) Holstein Cows during the PDP (Experiment 1)

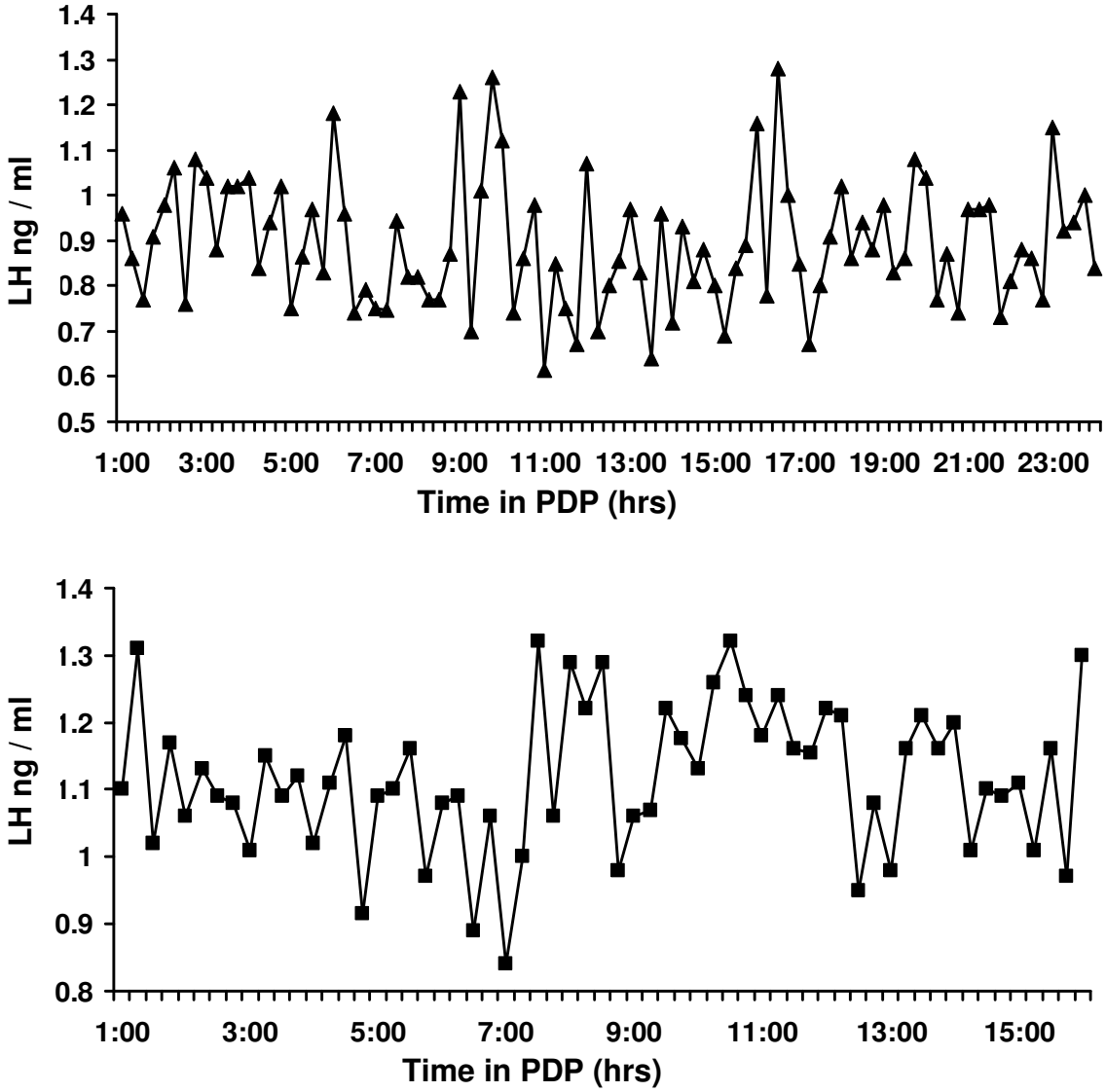


[a,b] Indicates a trend for a Difference (P<0.06) Between Groups at that Time.

The concentrations of LH were not different between groups. The total number of LH pulses during the PDP was lower in Hyperstimulated cows; however, the length of the PDP was shorter in Hyperstimulated cows. Overall, the number of LH pulses per hour

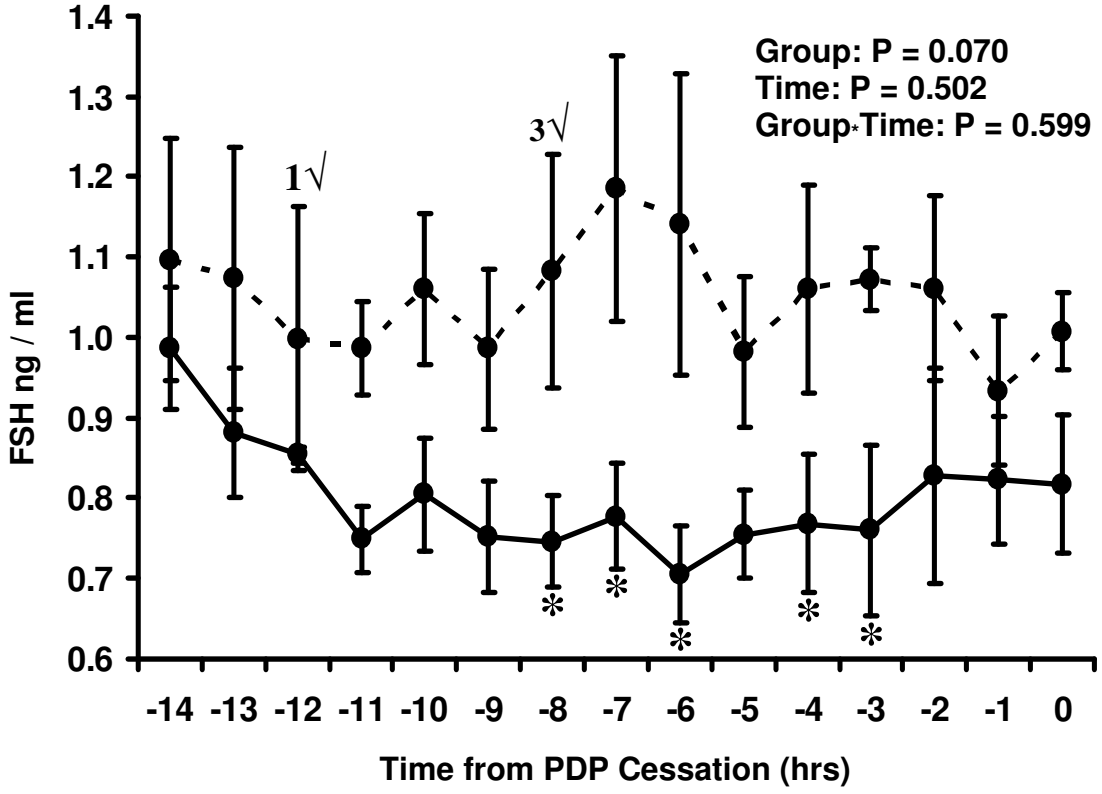
or pulse frequency did not differ between groups. The LH pulse pattern is illustrated using representative animals, one from each group, during the PDP in Figure 3.4.

Figure 3.4. Representative LH Patterns from Control (3409; ▲) and Hyperstimulated (3130; ■) Cows during the PDP



There was a trend toward an increased concentration of FSH in the Hyperstimulated Group (P = 0.066). The administration of FSH occurred at the same time points for all cows; however, the follicular development varied from animal to animal during the PDP. These data are normalized to the PDP cessation and the administration of FSH is indicated on the graph by a √**.** The number preceding the check indicates the number of cows that received FSH at that time.

Figure 3.5. The Concentration of FSH in Hyperstimulated (- -) or Control (—) Cows Normalized to the cessation of the PDP in Experiment 1



* Indicates a Difference (P<0.05) Between Groups at that Time

Discussion and Conclusion

Hypothesis One was rejected in this experiment because the pulse frequency of LH did not differ between groups nor did the average LH concentration. FSH concentrations tended to be elevated, not suppressed, in Hyperstimulated cows. Hypothesis Two was supported by these results because FSH administration resulted in a trend toward an increased number of large follicles (> 7- mm) at the initiation and cessation of the PDP an increased ovulation rate.

Cows' hyperstimulated with FSH experienced a shorter PDP and the largest follicle in the hyperstimulated follicular wave grew at an increased rate during the PDP compared to Controls. The second largest follicle in cows' hyperstimulated with exogenous FSH grew throughout the PDP and approached a 10- mm diameter by the cessation of the PDP while the second largest follicle in Controls did not approach 10- mm by the cessation of the PDP, perhaps indicative of follicular deviation or atresia of the subordinate follicle.

Multiple 7- mm follicles may have increased the competition among follicles and decreased the time required for the largest follicle to reach 10- mm. The trend toward an increased number of follicles > 7- mm at the cessation of the PDP may be indicative of prolonged window of opportunity for growing follicle to reach dominance in a hyperstimulated follicular wave. The trend toward an increase in the number of ovulations may have been due the presence of animals with relatively low hyperstimulatory responses (2 hyperstimulated cows only ovulated 4 follicles).

There was a trend toward increased concentrations of FSH in the Hyperstimulated Group and differences were identified at specific time points during the PDP. These differences may have been due to cross – reactivity between the administered porcine FSH and the endogenous FSH.

Experiment 1 employed the use of CIDR vaginal inserts to provide a regulated but sub – luteal source of Progesterone in the absence of an endogenous Progesterone source (corpus luteum). The sub – luteal Progesterone may have interfered with the production of Estradiol 17 -β and ultimately its suppressive impact on gonadotropins. Perhaps future work is needed to investigate the effects of varying concentrations of Progesterone on a hyperstimulated follicular wave. Additionally, the number of follicles an animal produces in a hyperstimulated follicular wave may have an impact on Estradiol 17 -β production and perhaps the change in endocrine dynamics may influence the proportion of high - quality oocytes released at ovulation and that are available for fertilization when used as part of an embryo collection protocol.

Cattle hyperstimulated with exogenous FSH experiences a follicular wave with more follicles at the initiation and cessation of the PDP. The pulse frequency was not affected by treatment but average FSH concentrations were elevated in hyperstimulated animals. The ovulation rate tended to be higher with FSH administration; however, the hyperstimulatory response was highly variable among cows. This experiment exposed some of the areas in need of further investigation to reveal possible explanations for the high variability in the hyperstimulatory response among cattle and from cycle to cycle.

CHAPTER FOUR

FOLLICULAR AND ENDOCRINE DYNAMICS ASSOCIATED WITH THE GROWTH OF THE LARGEST FOLLICLE FROM 6 – 10- MM IN HYPERSTIMULATED BEEF CATTLE WITH ONE OR ALL POTENTIAL DOMINANT FOLLICLES

Objectives

Administration of exogenous Follicle Stimulating Hormone (FSH) resulted in increased FSH concentrations and more follicles > 7- mm at the initiation and cessation of the perideviation period (PDP; 8-10 mm) in cattle. The pulse frequency and average concentration of Luteinizing Hormone (LH) was not affected by treatment (Chapter III). The estrogen dynamics associated with a hyperstimulated follicular wave remained undefined; therefore, the objective of this study was to determine if the number of dominant follicles in a hyperstimulated follicular wave influenced the endocrine profile during the PDP (6 – 10 mm). In order to further understand the follicular / endocrine interrelationship, Estradiol 17-β (E2), FSH, and follicular fluid (FF) E2 concentrations were evaluated during the PDP in beef cattle allowed to retain all or 1 follicle of a hyperstimulated follicular wave.

Perideviation Period (PDP)

The measured period of time from the first ultrasonographic detection of a 6- mm follicle until the first detection a 10- mm follicle in an engineered hyperstimulated follicular wave.

Hypothesis

Serum and follicular fluid concentrations of Estradiol 17 - β and FSH will be lower in hyperstimulated cattle allowed to retain 1 vs. all potential dominant follicles.

Materials and Methods

Estrous Synchronization

Estrus was synchronized in dry, open Angus or Angus - crossed cattle (n = 15) using a 25 mg intramuscular (IM) injection of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$ - ProstaMate®; Phoenix Scientific, Inc., St. Joseph, MO) to treat cows with corpra lutea (CL) at least 10 mm in diameter. Seven to 12 days post estrus, on Day 0, cows were treated with 25 mg IM injections of $PGF_{2\alpha}$, received a CIDR device (Eazi-breed CIDR; DEC International, NZ), and all follicles ≥ 4- mm were destroyed via ultrasound-guided trans-vaginal ablation with a 5 - MHz tranducer. Animals were kept on pasture with free choice water.

Treatment Protocol

On Day 1, cattle received a second 25-mg IM injection of $PGF_{2\alpha}$ to ensure luteal regression. Blood samples were collected and ovarian dynamics were documented ultrasonographically every 8 hrs starting on Day 2. Blood samples were collected from the Jugular vein and immediately transferred to 10 ml BD Vacutainer® Serum Separator Tubes (BD, Franklin Lakes, NJ) and refrigerated at 20°C for 8 hrs. Blood was then brought to room temperature and centrifuged for 20 min at approximately 12,000 x g. Serum was transferred to 20 ml glass vials with screw tops and stored at -20°C in a non-frost free freezer for future assay. FSH treatments (Sioux Biomedical, Sioux Center, IA,

Lot # 3097; 2.5 mg / ml) initiated 48 hrs after ablation (Day 2, concomitant with the emergence of a new follicular wave; Berfelt *et al*, 1994). A total of 15 mg of FSH were administered in 6 twice daily decreasing IM injections (2.0, 1.5, 1.0, 0.5, 0.5, 0.5 ml / injection).

Cattle were randomly divided into 2 groups. In the Single - Follicle Group (n = 8), only the first follicle that reached 6- mm was allowed to continue growing while other follicles (and subsequent 6-mm follicles) were ablated. CIDR implants were removed prior to and re-inserted immediately after follicular ablation. Ovarian ultrasound was performed every 8 hrs until a 10- mm follicle was detected. The 10- mm follicle was collected via trans – vaginal, ultrasound guided follicular aspiration and stored in glass vials and frozen at -20^{o}C for future assay.

In the Multi - Follicle Group (n = 7), all follicles were allowed to develop. CIDR implants were removed prior to and re-inserted immediately after follicular aspiration. Ovarian ultrasound was performed every 8 hrs until a 10- mm follicle was detected. When the largest follicle reached 10- mm, the 10 largest follicles were aspirated and the FF was stored for future assay. If the cow did not have 10 follicles, then the available follicles ≥ 8- mm were aspirated.

Serum Estradiol 17-β Radioimmunoassay

Serum E2 concentrations were measured using the Clemson University Endocrine Physiology Lab standard protocol for the detection of bovine Estradiol 17-β except the procedure was modified to allow for 2 ml of serum to be used for extraction and final

values were adjusted accordingly. The protocol was developed by Drs. D. M. Henricks and S. L. Gray (Appendix C; Breuel *et al*, 1988).

Briefly, the procedure began with the extraction of serum for standards, known E2 spikes, and serum samples through Baker disposable columns. Total counts tubes, non-specific binding tubes, standards, and spiked tubes underwent procedures similar to the procedures for samples and the variations were in accordance with the protocol found in Appendix C. Sample E2 was collected from the columns into borosilicate tubes with a methanol wash. Methanol was evaporated at 56°C. All tubes then received 100 µl $^3HE_2$ and 100 µl of E2 antiserum. Tubes were covered and refrigerated overnight at 4 – 8°C. Next, the tubes received 1 ml of charcoal suspension, were incubated in an ice bath for 20 min, and then centrifuged at 1,800 x g and 4°C for 10 min. Supernatant was decanted into scintillation vials and 4 ml of scintillation fluid was added to each vial. After inversion, the samples were kept in the dark for at least 3 hrs and then CPM were determined on a Beckman LS1800 scintillation counter. Any E2 concentrations with %CV greater than 20% were re-assayed in duplicate. Duplicates ≥ 2 standard deviations from the mean were discarded.

Follicular Fluid Estradiol 17-β Radioimmunoassay

Follicular fluid E2 concentrations were determined with the use of a Coat-A-Count Estradiol kit (Diagnostic Products Corporation, Los Angeles CA, Lot# TKE22 934). Samples were assayed in duplicate and the recommended procedure was followed. Samples (1 – 100 µl) whose concentrations were greater than the upper limit of the kit (1,800 pg / ml) were diluted until concentrations could be quantified, assayed again, and

subsequent values were corrected to account for the dilution factor. Any FF E2 concentrations with %CV greater than 15% were re-assayed in duplicate. Duplicates ≥ 2 standard deviations from the mean were discarded.

FSH Radioimmunoassay

Serum concentrations of FSH at hourly sampling intervals were measured in duplicate using a double antibody ^{125}I, 2 day competitive binding RIA procedure. The procedure (Appendix B) was based on the work of Dr. Parlow at the UCLA Medical Center's National Hormone and Peptide Program (Torrence, CA) and modified by Dr. S. Gray at the Clemson University Endocrine Physiology Lab (Clemson, SC). The NHPP supplied the bovine FSH for iodination and standards (AFP – 5332B), the first antibody (anti – oFSH – 1 A.S., RIA AFPC – 5288113Rb), and the Goat Anti – Rabbit IgG 2nd Antibody (Rockland Anti – Rabbit IgG Lot # 16528). Any FSH concentrations with %CV greater than 15% were re-assayed in duplicate. Duplicates ≥ 2 standard deviations from the mean were discarded.

Data Analysis

Windows Single factor analysis of variance (ANOVA) was used to evaluate differences in serum and FF E2, FSH concentrations, and follicular dynamics between groups. SAS 9.1 ANOVA was used to detect Group, Time, and Group by Time interactions. Fisher's Least Significant Difference Test was used to assess differences at specific times.

Assay Efficiencies

Five serum E2 assays were conducted with intra-assay %CV ranging from 2.3% to 3.3% and an inter-assay %CV of 2.8%. Follicular fluid E2 concentrations were determined in 3 assays resulting in an intra-assay %CV ranging from 2.9 – 7.1% and an inter-assay %CV of 4.4%. Three FSH assays were used and had intra-assay %CV from 9.4% to 11.0% and an inter-assay %CV of 10.4%.

Results

The serum E2 concentrations were elevated while FSH was suppressed when a 10- mm follicle was detected. The E2 and FSH concentrations for all diameter ranges are in Table 4.1.

Table 4.1. Serum E2 (pg/ml) and FSH (ng/ml) Concentrations at Specific Follicular Diameters in Beef Cattle Allowed to Retain All or 1 Follicle (Experiment 2)

	E2 pg / ml			FSH ng / ml		
Largest Follicle (mm)	**Single – Follicle Group**	**Multi – Follicle Group**	**P-value**	**Single – Follicle Group**	**Multi – Follicle Group**	**P-value**
6.0 - 6.9	5.14 ± 0.99	5.15 ± 1.11	0.993	1.23 ± 0.06	1.44 ± 0.17	0.159
7.0 - 7.9	4.72 ± 1.16	3.40 ± 0.72	0.345	1.19 ± 0.12	1.03 ± 0.07	0.249
8.0 - 8.9	4.14 ± 1.42	7.22 ± 2.90	0.442	1.03 ± 0.07	0.93 ± 0.05	0.408
9.0 - 9.9	5.49 ± 0.77	8.89 ± 4.78	0.249	1.00 ± 0.04	0.96 ± 0.16	0.552
10.0	4.38 ± 0.82	10.46 ± 2.43	**0.057**	1.01 ± 0.07	0.66 ± 0.23	**0.045**

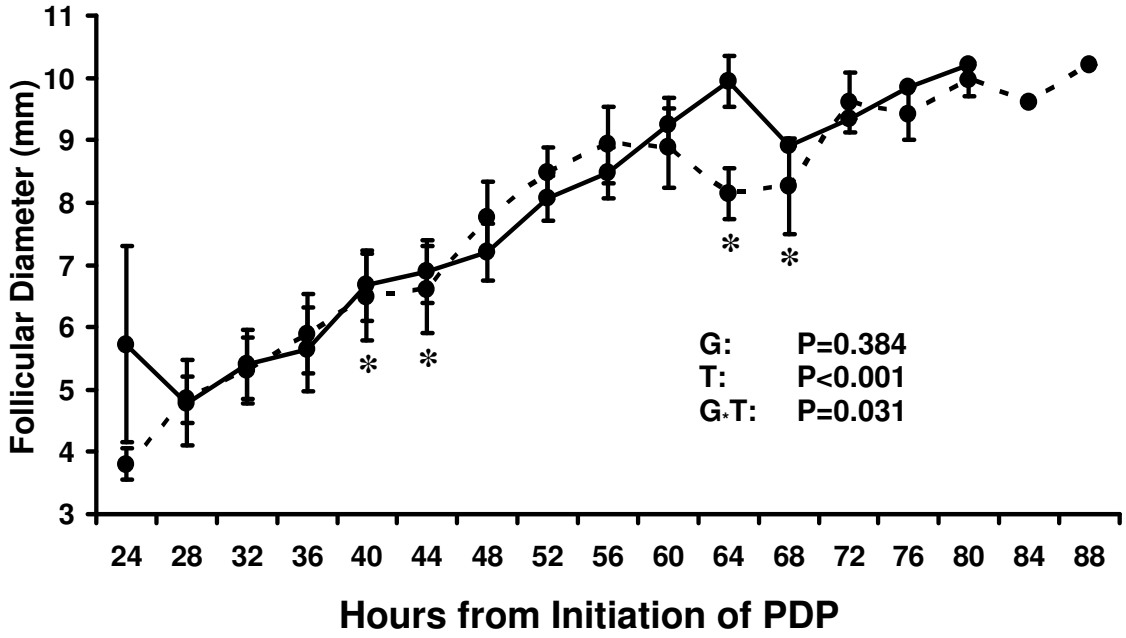
Total FF E2 concentrations were determined, divided by the number of follicles collected to calculate the amount of E2 produced per follicle, and analyzed with ANOVA (Table 4.2). One cow in the Multi – Follicle Group only produced 2 follicles ≥ 6- mm and the resulting follicular fluid data was not incorporated into the follicular fluid analysis.

Table 4.2 Follicular Fluid E2 Concentrations (ng/ml) at Cessation of PDP in Beef Cattle Allowed to Retain all or 1 Follicle (Experiment 2)

	Single – Follicle Group	Multi – Follicle Group	P-value
Number of Aspirated Follicles	1.0 ± 0.0	10.0 ± 0.0	<0.001
E2 / Follicle (ng / ml)	26.7 ± 31.2	17.9 ± 74.8	0.326
Total E2 (ng / ml)	26.7 ± 31.2	89.0 ±28.0	0.076

The largest follicle(s) growth rates were compared between groups. The number of follicles retained in a hyperstimulated follicular wave did not impact the duration of the PDP and follicles grew at a similar rate. The growth profile of the largest follicle in each group are summarized in Figure 4.1

Figure 4.1 Follicular Development (mm) of the Largest Follicle from the Single - Follicle Group (--) and the Multi - Follicle Group (—) in Experiment 2



* Indicates a difference (P<0.05) between groups at that hour.

Discussion and Conclusions

Results of this study supported the hypothesis because serum Estradiol 17-β increased and FSH decreased in the Multi-Follicle group when the largest follicle reached 10 mm (PDP cessation). There was also a trend for an increase in follicular fluid E2 at the cessation of the PDP in the Multi - Follicle Group compared to the Single-Follicle Group. Serum E2 gradually increased while FSH declined more severely in the Multi – Follicle Group compared to the Single – Follicle Group.

This study demonstrated that an increase in the number of retained follicles in a hyperstimulated follicular wave increased serum E2 concentrations and decreased FSH concentrations at the cessation of the. The delay in serum E2 elevation may be indicative

of a prolonged endocrine deviation system induced by FSH administration and increased follicular competition for dominance

There was a trend toward an increase in FF E2 production in the Multi – Follicle Group. Additionally, there was a 10- fold increase in the number of follicles and follicles > 8.5- mm should have E2 production capacity (Ginther *et al*, 1996), but there was not a significant difference in total E2 production. There was no difference in E2 production per follicle perhaps indicative of a compensatory mechanism among follicle to produce a systemic threshold amount of E2. Another possibility is that E2 was produced from select follicles regardless of size. If true, then perhaps the E2 producing follicles suppress other large follicles from contributing to E2 production.

In Experiment Two, CIDR vaginal inserts were used to provide a regulated and sub – luteal Progesterone source. The Progesterone released from these inserts is enough to inhibit estrus in cattle (Chenault *et al*, 2003). Perhaps these sub – luteal doses played role in suppressing E2 production especially in the Multi – Follicle

The differences in growth rates could be due to altered competition among follicles in the Single - Follicle Group. Furthermore, each animal in the Single - Follicle Group was trans - vaginally ablated an average of 3.5 times during the PDP. This physical disruption of follicular dynamics and the local environment may have impacted growth of the largest follicle. This study concluded that an increase in the number of retained follicles in a hyperstimulated follicular wave influenced the endocrine profile during the PDP (6 – 10 mm) by increasing serum and FF E2 concentrations and suppressing FSH concentrations.

CHAPTER FIVE

OVERALL CONCLUSIONS, DISCUSSION, AND FUTURE WORK

Results from these experiments indicated that cattle hyperstimulated with Follicle Stimulating Hormone (FSH) experience several changes in endocrine dynamics and follicular growth profiles during the PDP. Furthermore, the numbers of follicles present before and during the PDP in hyperstimulated animals impacted the endocrine profile.

The administration of FSH tended to increase the number of follicles > 7- mm at the initiation and the cessation of the PDP and ultimately the number of ovulations. Hyperstimulated cows experienced a faster growth rate of the largest follicle during the PDP and required less time to develop a 10- mm follicle. The administration of FSH did not alter the LH pulse frequency. When hyperstimulated cattle with either 1 or all potential dominant follicles were compared, there were transient increases in circulating FSH concentrations and a trend toward higher average FSH concentration in the Multi – Follicle Group. At the cessation of the PDP, serum E2 concentrations were elevated in the Multi – Follicle Group and may have contributed to the suppression of FSH. Follicular fluid collected at the cessation of the PDP revealed a trend for increased E2 production in the Multi – Follicle Group, but the calculated estimate of E2 production per follicle was not different between groups.

Results from these experiments indicate that ovarian hyperstimulation with FSH may prolong the deviation process. Endocrine dominance may be a better indicator of follicular maturation and perhaps oocyte quality and capacity for fertilization than diameter measurements alone. Furthermore, while results from Experiment Two

illustrated a trend toward increased total FF E2 in the Multi – Follicle Group, a compensatory mechanism of follicles working in concert to produce E2 may exists; however, results did not show a direct relationship between the number of large follicle and the FF E2 concentrations. It is important to note that the use of CIDR vaginal inserts may have impacted the E2 production in large follicles by providing a sub – luteal Progesterone dose.

Follicular fluid collected at the end of the PDP in the second experiment was pooled and E2 production per follicle was calculated based upon the number of follicles collected. Therefore, future experiments utilizing the collection of follicular fluid from individual follicles of specific diameters, perhaps ranging from 6 – 12 mm, are necessary to understand the contributions from individual follicles in a hyperstimulated follicular wave.

A future experiment to investigate the effects of hyperstimulated follicular waves with a variety of numbers of follicles is warranted. There may a relationship between the endocrine dynamics in these waves and the oocyte quality and capacity to yield high quality embryos. Experiments aimed at the determining the difference in the deviation mechanism, based on follicular size and endocrine markers, in hyperstimulated follicular waves may reveal meaning information on follicular maturation. The role of Progesterone and E2 and their interrelationship with oocyte viability in a hyperstimulated follicular wave are also in need of further investigation. The combined information from these studies may help modify current ovarian hyperstimulatory protocols and assist the cattle industry in improving high quality embryo yields.

APPENDICES

Appendix A

Clemson University Endocrine Physiology Lab
Radioimmunoassay Procedure for Bovine Luteinizing Hormone

Reagents:

1. 0.01 M Phosphate Buffered Saline (PBS) (2 liters)
   a. 3.43 g Sodium Phosphate (monobasic)
   b. 17.73 g Sodium Chloride
   c. 1.95 L Distilled – Deionized Water
   d. 0.2 g Thimersol
   Adjust pH to 7.5

2. 0.01 M Phosphate Buffered Saline with 1% Bovine Serum Albumin (1% PBSA)
   a. 1 g Bovine Serum Albumin (BSA)
   b. 100 ml 0.01 M PBS Buffer

3. 6% Polyethylene Glycol (PEG)
   a. 6 g Polyethylene Glycol
   b. 100 M 0.01 M PBS buffer
   Adjust pH to 7.5
   Store at 4◦C

4. 0.05 M Ethylenedinitrilo-tetraacetic Acid (EDTA)
   a. 1.9 g EDTA
   b. 100 ml 0.01 M PBS buffer
   Adjust to pH 7.5
   Store at 4◦C

5. Normal Rabbit Serum (NRS)
   Store Frozen

6. NRS / EDTA / PBS (1 : 200 NRS : 0.05 M EDTA / PBS)
   a. 100 µl of NRS
   b. 20 ml of 0.05 M EDTA / PBS

7. Bovine LH (Bolts)
   Weigh out and solubilize to get 0.1 µg of protein / µl of 0.5 M PO_4 buffer.
   Use for iodination.

8. Radioiodinated bLH (See radioiodination procedure)
   Dilute to approximately 10,000 – 12,000 / µl using 1% PBSA

9. First Antibody (in rabbit serum) (DJB – bLHAB)
   Dilute 1 : 60,000 with NRS / EDTA / PBS

10. Second Antibody (sheep anti – rabbit serum)
    Dilute 1 : 15 with 1% PBSA

11. Transfer Solution
    KI – 10 mg / ml in 16% Sucrose in water

12. Rinse Solution
    KI – 10 mg / ml in 8% Sucrose in water

13. 0.5 M PO_4 buffer
    a. Disodium phosphate 2.9182 g / 50 ml water
    b. Monosodium Phosphate 0.5727 g / 50 ml water
    Mix together
    Adjust pH to 7.5

Sephadex Column Preparation

1. Swell P – 60 in 0.01 M PBS buffer overnight (2 g / 50 ml of buffer)
2. Place under vacuum for approximately 2 hours.
3. Use 10 ml disposable pipette for column (cut top off column to have wider opening)
4. Place small amount of glass wool in base of column.
5. Place 1-2 inches tubing on tip of column with pinch cock to stop flow.
6. Add small amount of slurry 1-2 inches to column and allow to settle.
7. Unclamp column and fill column with slurry allowing to settle as it runs through.
8. Rinse column with 0.01 M PBS buffer (pH 7.5) 3 -4 ml.
9. Rinse column again with 1% PBSA buffer (pH 7.5) 3 – 4 ml.
10. Rinse column again with 0.01 M PBS buffer (pH 7.5) 10ml.

Prolactin Iodination Procedure

1. Use 5 μg protein for iodination – place in iodination vial.
2. Add 30 μl of 0.5 M PO_4 buffer to reaction vial.
3. Draw 10 μl of 0.5 M PO_4 first followed by 10 μl of ^{125}I into a Hamilton syringe and add this to the reaction vial.
4. Add 20 μl Chloramine – T (20mg / 10 ml 0.01 M PBS buffer with pH 7.5)
5. React for 60 – 65 seconds.
6. Add 50 μl of sodium metabisulfate (25 mg / 10 ml 0.01 M PBS with pH 7.5).
7. Mix for 20 seconds.

8. Add 100 µl transfer solution to reaction vial and transfer the contents to the column. Note: First remove buffer layer from column head.
9. Add 100 µl of 1% PBSA to the reaction vial.
10. Add 70 µl of rinse solution to the reaction vial.
11. Transfer this onto the column.
12. Open the column and start collecting 25 fractions in 12 x 75 mm tubes containing 100 µl of 1% PBSA. Elute the column with the 0.01 M PBS buffer (pH 7.5). Collect 1 ml in first tube. Collect 0.5 ml in all other tubes.
13. Count 10 µl fractions of each tube for 1 minute.

Luteinizing Hormone Procedure

First Day

1. Add 200 µl of each of the following standards into duplicate 12 x 75 mm tubes.

10 ng LH / 200 µl	=	50 ng LH / ml
5 ng LH / 200 µl	=	25 ng LH / ml
2.5 ng LH / 200 µl	=	12.5 ng LH / ml
1.25 ng LH / 200 µl	=	6.25 ng LH / ml
0.625 ng LH / 200 µl	=	3.125 ng LH / ml
0.313 ng LH / 200 µl	=	1.5625 ng LH / ml
0.156 ng LH / 200 µl	=	0.7812 ng LH / ml
0.078 ng LH / 200 µl	=	0.39 ng LH / ml

   Prepared from serial dilution of 0.1 µg LH / µl stock solution.
   Dilute with 1% PBSA.
2. Add 200 µl of each unknown into duplicate 12 x 75 mm tubes.
3. Total bound tubes (TB) and Nonspecific binding tubes (NSB) receive 200 µl of 1% PBSA. Total count tubes (TC) receive nothing.
4. All tubes except TC and NSB tubes receive 200 µl of the first antibody (DJB – bLHAB) (1: 60,000 dilution). NSB tubes receive 200 µl of PBSA and TC tubes receive nothing.

Second Day

5. 100 µl of ^{125}I labeled bLH at 10,000 – 12,000 cpm / 100 µl is added to all tubes.

Third Day

6. All tubes except TC tubes receive 200 µl of second antibody (1 : 15 dilution) and 1 ml of 6% PEG. Let sit 15 minutes.
7. Centrifuge for 25 minutes at 2500 RPMs.
8. Decant supernatant (except TC) and allow to dry upside down for 5 minutes.
9. Count tubes in gamma counter for 1 minute.

Appendix B

Clemson University Endocrine Physiology Lab
Radioimmunoassay for Bovine Follicle Stimulating Hormone Procedure

Reagents should be prepared in accordance with the directions listed in the bLH Procedure.

First Day

10. Add 100 µl of each of the following standards into duplicate 12 x 75 mm tubes.
    20 ng LH / ml
    10 ng LH / ml
    5 ng LH / ml
    2.5 ng LH / ml
    1.25 ng LH / ml
    0.62 ng LH / ml
    0.31 ng LH / ml
    Dilute with 1% PBSA.
11. Add 100 µl of each unknown into duplicate 12 x 75 mm tubes.
12. Total bound tubes (TB) and Nonspecific binding tubes (NSB) receive 100 µl of 1% PBSA. Total count tubes (TC) receive nothing.
13. All tubes except TC and NSB tubes receive 100 µl of the first antibody (DJB – bLHAB) (1: 175 dilution). NSB tubes receive 100 µl of PBSA and TC tubes receive nothing.
14. 50 µl of ^{125}I labeled bFSH at 15,000 CPM is added to all tubes.
15. Shake tubes and incubate at room temperature for 24 hours.

Second Day

16. All tubes except TC tubes receive 1 ml of 6% PBS / PEG with a 1 : 300 dilution of second antibody, goat anti-rabbit GG.
17. All tubes except TC tubes receive 100 µl of 1.5% NRS in PBS.
18. Incubate at room temperature for 4 hours.
19. Centrifuge for 30 minutes at 18,000 g.
20. Decant supernatant (except TC) and allow to dry upside down for approximately 5 minutes.
21. Count tubes in gamma counter for 1 minute.

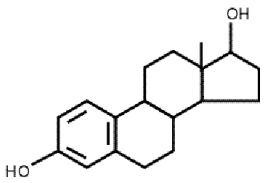
Appendix C

Estradiol-17β Extraction and Radioimmunoassay of Bovine Serum

Endocrine Physiology Laboratory
Clemson University

SOP NO. M06 DATE: 7/31/97 REVISION: 01/27/07

Structure: 1,3,5(10)-ESTRATRIEN-3,17-Diol(17 Estradiol)



The following chemicals used in this method are flammable: *methanol, acetone and ethanol*
The following chemicals may pose a health risk, Read MSDS prior to use: *methanol, acetone and ethanol*
The following chemicals are corrosive: *formic acid, hydrochloric acid and sodium hydroxide*
Only use these chemicals with adequate ventilation.
Training in radiation safety is required prior to operating this method.
Always wear protective gear (gloves, lab coat, eye protection)

Developed by: Drs. Donald M. Henricks and Sandra L. Gray, Endocrine Physiology Laboratory, Animal and Veterinary Sciences Department, Clemson University, Clemson, SC 29634-0311.

1.0 Scope

This analytical method describes the procedure for extracting and quantifying the concentrations of 17β-estradiol (E2) in bovine serum.

2.0 Principle

Estradiol concentrations in serum are measured by extracting the steroid hormone from the serum and concentrating it by reverse phase chromatography followed by liquid phase radioimmunoassay (RIA). In the chromatography step, an aliquot of the serum is placed on a mini-octadecyl column and the estradiol is eluted off in a manner to exclude the bulk of the other compounds present. The RIA meets the criteria of accuracy, sensitivity, precision and specificity to provide a reliable practical means of measuring estradiol-17β in this fluid.

3.0 Equipment

3.1 Pipets
- 3.1.1 Eppendorf Adjustable 500 µl - 2500 µl
- 3.1.2 Gilson Pipetman digitals
- 3.1.3 Nichiryo Repeating Pipettor
- 3.1.4 Barnstead/Labindustries Repipet II
- 3.1.5 Wiretrols (Drummond Co.) 5 µl, 10 µl, 20 µl, 25 µl, 50 µl, 100 µl
- 3.1.6 Oxford, Barnstead bottle pipettors
- 3.1.7 Hamilton repeating 100 µl dispenser (clicker)
- 3.1.8 Labindustries bottle repipettor

3.2 General laboratory instruments
- 3.2.1 Fisher Scientific A-200D balance
- 3.2.2 Corning PC-351 hot plate stirrer
- 3.2.3 Corning Model 7 pH meter
- 3.2.4 Thermolyne Dri-Bath
- 3.2.5 'Baker' spe-24G extraction system (Baker Scientific Co.)
- 3.2.6 Speedaire air compressor
- 3.2.7 Beckman LS1800 scintillation counter
- 3.2.8 Model J-6B centrifuge
- 3.2.9 Thermolyne Maxi Mix II

3.3 General laboratory glassware
- 3.3.1 Disposable glassware used wherever practical.
- 3.3.2 13 x 100 mm and 12 x 75 mm borosilicate glass culture tubes
- 3.3.3 Reusable glassware is cleaned according to strict procedure for cleaning glassware.
- 3.3.4 7 ml polyethylene scintillation vials

4.0 Reagents

4.1 Organic solvents
- 4.1.1 Methanol (nanograde)
- 4.1.2 Acetone, histological grade
- 4.1.3 Ethanol - 95% (glass distilled)
- 4.1.4 Perkin-Elmer (formerly Packard) Ultima Gold LSC-Cocktail or equivalent

4.2 Buffers (See attachment for recipe)
- 4.2.1 AR sodium chloride
- 4.2.2 Sodium phosphate, dibasic
- 4.2.3 Sodium phosphate, monobasic (ACS grade)
- 4.2.4 Thimerosal
- 4.2.5 Knox gelatin
- 4.2.6 Sodium hydroxide (ACS grade)
- 4.2.7 Hydrochloric acid (ACS grade)
- 4.2.8 Deionized water (Continental water/Millipore system)

4.3 'Baker Analyzed' formic acid (88%) or equivalent

4.4 Calf serum (Atlanta Biologicals, Inc.) or equivalent

4.5 'Baker Analyzed' spe octadecyl (C_{18}) 3 ml LD disposable columns or equivalent

4.6 17β-Estradiol from Steraloids, Inc.
- 4.6.1 Standard stock: Stored according to manufacture's recommendations.
- 4.6.2 Stock standard solution: Dissolve an accurately weighed quantity of 17β-estradiol (at least 100 ug) in distilled ethanol and dilute to volume in volumetric flask with distilled ethanol. Stable indefinitely at < -10°C.
- 4.6.3 Standards are serially diluted in ethanol from the 1 µg/ml stock standard solution to include the quantities of 3.1, 6.25, 12.5, 25, 50, 100, 200 and 400 pg. Standards are stable for approximately 30 days when stored at approximately 4^{o} C

4.7 Estradiol-17, [2,4,6,7-^{3}H(N)] 250 µCi from PerkinElmer (formerly New England Nuclear)
- 4.7.1 Initial tritiated stock: Store at < -10^{0} C or manufacturer's recommendations.
- 4.7.2 Intermediate stock: Mix initial tritiated stock with distilled ethanol and dilute in 25 ml volumetric flask with addition of distilled ethanol for a concentration of 250 µCi/25 ml. Solution is stable for 12-24 months at < -10^{0} C.

4.7.3 Assay stock of tritiated solution: Mix approximately 1 ml of stock tritiated solution with 100 ml of PBSMG Buffer for 8,000 - 12,000 cpm in 100 µl. Solution stable for approximately one month at approximately 4-8°C.

4.8 Estradiol-17β Antiserum (Lot #0205041673)
(Gift from Eli-Lilly, Dr. Norm Mason, Indianapolis, Indiana)

4.8.1 Stock antiserum solution of 1:2 dilution in PBSMG buffer stored indefinitely at < -10°C.

4.8.2 Working stock antiserum solution of 1:10 dilution stored indefinitely at approximately 4°C.

4.8.3 Working stock antiserum solution of 1:10 is diluted in PBSMG buffer to give approximately 40% B_0/TC. This dilution should be made no more than one hour prior to use.

4.9 Activated charcoal

4.9.1 Charcoal suspension of 250 mg/100 ml PBSMG buffer is allowed to stir for 2-3 hours prior to use.

4.9.2 Charcoal suspension may be made one day in advance and stored in refrigerator at approximately 4-8°C until needed. When stored overnight, suspension is allowed to stir at least 20 minutes prior to use.

5.0 Quality Control

5.1 Pools

5.1.1 A shipping stability pool of serum containing a known steroid concentration may be included with each shipment of serum coming from other facilities. This pool is assayed at least once with the samples received in the same shipment (obtained from the animal study site).

5.1.2 Incurred serum pools may be stored in the freezer at the laboratory. concentrations in aliquots of these pools are measured at regular intervals during the course of a study.

5.1.3 Daily spike pools to include a blank, low, medium, and high pool are included in each assay. Pools are made by adding known spike concentrations from standards to blank calf serum. Ethanol is added to blanks at an equivalent volume used for spikes.

5.2 $^3HE_2$ recovery from column extraction

In each assay, recovery of $^3HE_2$ is measured by addition of a known amount of ^3H E_2 to calf serum. Serum samples are extracted and concentrated by eluting from mini-octadecyl columns into scintillation vials. Total count (TC) samples, which are actually calf serum without

any ^{3}H E_2, are also extracted and eluted in the same way. $^{3}HE_2$ is added later to TC vials.

6.0 Procedure

6.1 Preparation and protein precipitation of serum curve, blank, daily spike pool, $^{3}HE_{22}$ recovery (REC), recovery total counts (TC) and test samples.

6.1.1 Pipet required volume (0.1-1.1 ml) of control serum for serum curve, blanks, daily spikes, $^{3}HE_2$ recovery (REC) , and TC samples into appropriately labeled 13 x 100 mm borosilicate culture tubes. Samples are pipetted in duplicate except for ^{3}H recovery which is pipetted in triplicate

6.1.2 Add 100µl $^{3}HE_2$ (about 10,000 cpm) to REC tubes.

6.1.3 Add 100µl of the appropriate standards (warmed to ambient temperature) to the daily spike samples. Add 100 µl ethanol to blank tubes.

6.1.4 Biologically bound steroids in the serum are freed for extraction by adding an equivalent volume of 0.1 N formic acid to the serum in the 13 x 100 mm culture tubes and vortexing.

6.1.5 Centrifugation of serum sample with formic acid at 1800 x g (3000 rpm) for 5 minutes may be required to pellet proteins.

6.2 Column preparation and sample extraction method

6.2.1 Serum samples extracted using 'Baker' spe-24G system with Baker spe disposable columns (octadecyl C_{18} 3ml LD or equivalent). Note: Vacuum pump is used to pull all solvents and samples through column. Each column may be used to process four samples, and is then thrown away.

6.2.2 Octadecyl columns are first conditioned as follows:

6.3.2.1 Rinse with approximately two column vol of methanol.

6.3.2.2 Rinse with approximately two column vol of deionized water.

6.3.2.3 Rinse with approximately one column vol 20:80 acetone:water.

6.3.2.4 Leave under vacuum for approximately five minutes.

6.3.2.5 Rinse with approximately one column vol of methanol.

6.3.2.6 Rinse with approximately one column vol of deionized water. Turn vacuum off. . Keep columns moist with deionized water if needed.

6.3.2.7 Add 1.0 ml of calf serum to each column and allow to run through.

6.3.2.8 Add one column vol 20:80 acetone:water to columns.

6.3.2.9 Keep in vacuum for 3 minutes. Note: Conditioning step is required only once with each set of columns.

6.2.3 Extraction of serum curve, blank, daily spikes, $^3HE_2$ recovery (REC), recovery total counts (TC) and test samples

6.2.3.1 Rinse columns with approximately one column vol methanol.

6.2.3.2 Rinse columns with approximately one column vol deionized water. Turn vacuum off. Keep columns moist with more deionized water if needed.

6.2.3.3 Add prepared serum curve, blank, daily spike pool, REC, TC, and test samples to columns and turn on vacuum. If possible, add prepared serum to columns leaving protein pellet in the tube. Use Note-to-file to explain calculations. Note: TC and REC samples are heated at approximately 45°C for 5 minutes before pipetting on columns

6.2.3.4 Add approximately one column vol 20:80 acetone:water to columns, while vacuum running.

6.2.3.5 Keep in vacuum for 3 minutes.

6.3.3.6 Turn off vacuum; dry column tips by wiping with a Kimwipe.

6.3.3.7 Insert 12 x 75 mm glass tubes in test tubes holder and place in vacuum box under columns. Note: Scintillation vials are inserted in test tube holder for REC and TC samples

6.3.3.8 Add 0.3 ml methanol to columns and turn on vacuum.

6.3.3.9 Repeat 6.2.3.8 once.

6.3.3.10 Remove tubes or vials containing 0.6 ml methanol and extracted steroid from system and test tubes holder.

6.3.3.11 Repeat procedures in 6.3 for all serum samples. Note: The same columns may be used approximately four times or until particulate material from the serum samples prohibits easy flow through the columns.

6.4 Final steps in determination of estradiol (setting up the RIA)

6.4.1 Add 100 µl appropriate standards to methanol extracts for standard curve; add 100 µl ethanol to total binding tubes.

6.4.1 Evaporate to dryness under a steady stream of air at approximately 56°C on heating block, the methanol from extracted samples and methanol from standard curve points (including NSB, TB, and TC).

6.4.2 Add 100µl PBSMG buffer to REC vials. Add 100µl $^3HE_2$ to TC vials. Then see steps 6.4.11 and 6.4.12 for these vials.

6.4.3 Using Hamilton repeating pipet (clicker), add 100 µl $^3HE_2$ to all tubes in assay.

6.4.4 Using Hamilton repeating pipet (clicker), add 100 µl of E_2 antiserum diluted to give approximately 40% B_0/TC to all tubes except the NSB (nonspecific bound) and TC tubes. To NSB tubes, add 100 µl of PBSMG buffer.

6.4.5 Shake tubes gently, cover and refrigerate overnight at approximately 4-8°C.

6.4.6 After overnight incubation, place racks of tubes in ice bath.

6.4.7 Using a repeating pipettor bottle, add approximately 1.0 ml of charcoal suspension stirred in ice bath to all tubes in assay except TC (total count tubes). To the TC tubes, add 1.0 ml PBSMG buffer. Note: It is important that charcoal be well stirred, and cold (approximately 4°C). Charcoal must be added in rapid succession to all tubes -- no more than 2 minutes from beginning sample to end sample.

6.4.8 Allow samples to incubate with charcoal in ice bath for 20 minutes.

6.4.9 Centrifuge samples at 4°C at 1800 x g for 10 minutes.

6.4.10 Pour supernatant off charcoal pellets into corresponding scintillation vials. Note: It is important that supernatant be poured off immediately after centrifugation stops and that there be no interruption once the pouring off is begun.

6.4.11 Add 4 ml of scintillation fluid to all scintillation vials, cap tightly, and mix by inversion.

6.4.12 Dark adapt for at least three hours before counting radioactivity on scintillation counter.

7.0 Acceptance criteria for E-2 assay

7.1 The $^{3}HE_2$ recovery from column extraction must be greater than 80%.

7.2 The blank serum should read below the established LOQ for the assay.

7.3 Mean concentrations from at least two of the three spike pools must read ± 20% of the expected value. Examples of pool concentrations that could be used are:

QC Pool	Mean Value	± 20% of Mean Value (pg/ml)
Low	12.5	10-15
Mid	50	40-60
High	200	160-240

8.0 Acceptance criteria for individual test samples

8.1 The CV between calculated concentrations of replicates must be ≤ 20%.

8.2 When extracting 1 ml replicates, calculated concentrations for the test samples must fall within the range of the standard curve, 0-400 pg. The

cpm for all samples should be ≥ 500 cpm. Samples having a steroid concentration greater than 400 pg or cpm < 500 will be reassayed, using a smaller serum aliquot to ensure the sample concentration will fall within standard curve range.

8.3 If one replicates of the extracted sample does not fall within the range of the standard curve, the test sample will be reassayed. A sample will be reassayed a maximum of two times. Concentrations of steroids in samples not meeting acceptance criteria after three attempts will not be reported.

9.0 Notes

9.1 Assay LOQ

Standard curves for the assay covered the range 3.13 – 400 pg. Assay LOQ usually falls between 6 -12 pg.

9.2 Accuracy and precision of assay

9.2.1 Accuracy and precision are based on assay meeting three criteria

9.2.1.1 Consistency in standard curves between assays

9.2.1.2 % CV ≤ 20 between sample replicates

9.2.1.3 Replication of pool values between assays. Note: The assay is accepted if 2 of the 3 pools in the assay read within 20% of their expected value and/or if the pools that read in the range of the unknown samples are reading within 20% of their expected values.

9.2.2 Any assays in doubt are rerun in total.

9.2.3 Samples in doubt (poor replication or unexpected values) are repeated.

9.2.4 Construction of the standard curve by the data calculation software must be based on at least five standard curve points.

9.3 Details to be described in specific study protocols if applicable

9.3.1 Concentrations and preparation of standards

9.3.2 Analytical matrix and assay volume

9.3.3 Quality control pool preparation

9.3.4 Assay, QC samples and test sample acceptability criteria

Attachment

PBSMG buffer (0.1 M, pH 7, 0.1% gel)

Solution A
27.6 gm $NaH_2PO_4 \cdot H_2O$/liter (0.2 M)
Solution B
53.6 gm $Na_2HPO_4 \cdot 7H_2O$/liter (0.2 M)

Add 195 ml Solution A to 305 ml Solution B.
Add 9 gm NaCl.
Add 0.1 gm sodium merthiolate (Thimerosal).
Add 1 gm Knox gelatin; stir and heat until gelatin is dissolved.
Bring up final volume of 1 liter with deionized, distilled water.
Adjust pH to 7.0 with NaOH or HCl.

Procedure for washing glassware

Soak used glassware in Alconox or equivalent soap solution overnight. Soak radioactive glassware in detergent solution.

Appropriately sized glassware may be washed in automatic dishwasher. Glassware which has been soaked in detergent does not need additional detergent added to automatic dishwasher.

All glassware must be rinsed twice with distilled water, then once with distilled EtOH, and last with distilled acetone. Exception: Do not rinse plastics of polycarbonate with acetone.

Invert washed glassware onto clean paper on drying cart and allow to dry at room temperature. Replace all glassware to covered cabinets and shelves as soon as possible.

Appendix D

Society for the Study of Reproduction Abstract 2007

Endocrine Dynamics Surrounding Dominant Follicle Selection in Cattle

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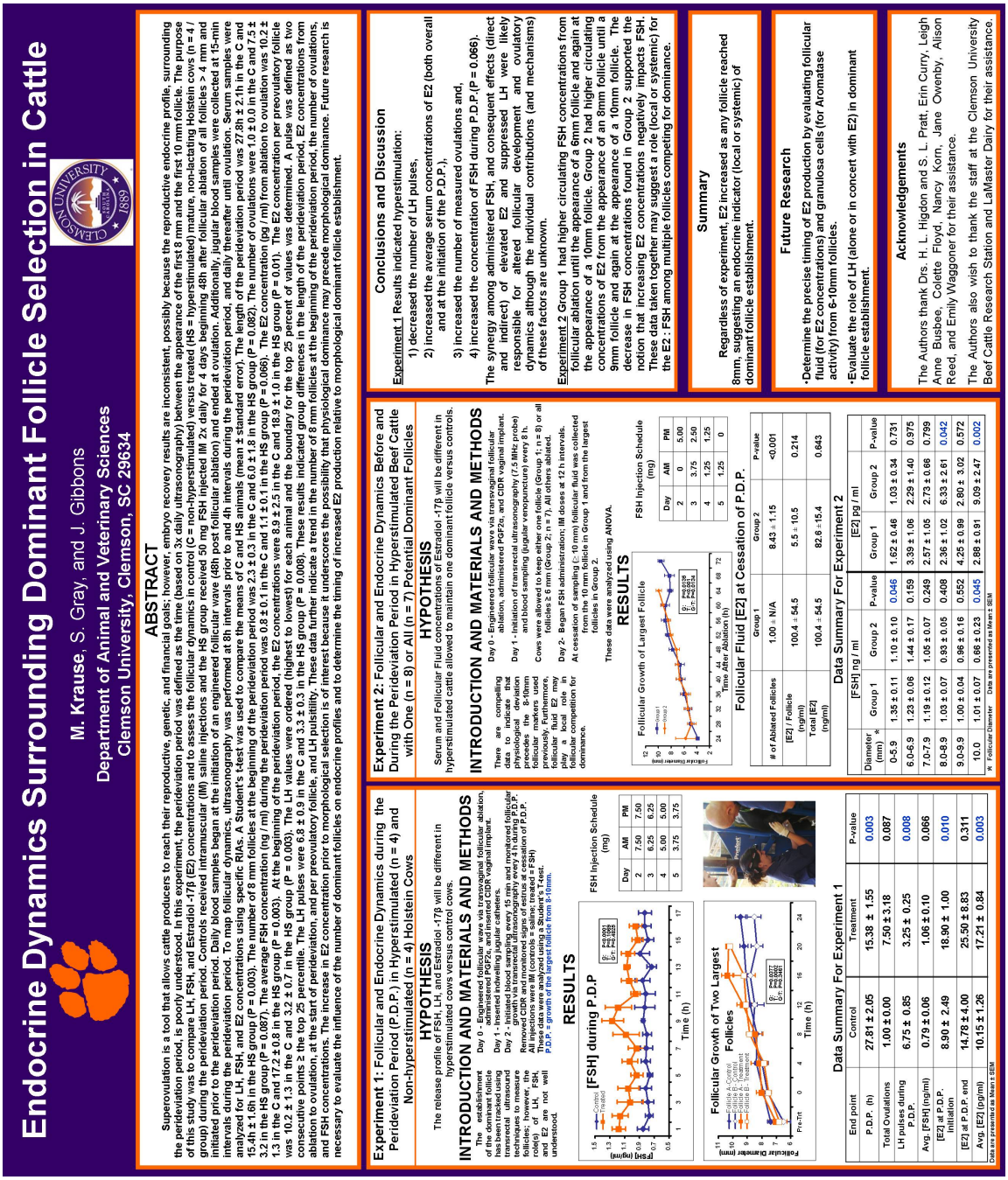
Superovulation is a tool that allows cattle producers to reach their reproductive, genetic, and financial goals; however, embryo recovery results are inconsistent, possibly because the reproductive endocrine profile, surrounding the perideviation period, is poorly understood. In this experiment, the perideviation period was defined as the time (based on 3x daily ultrasonography) between the appearance of the first 8 mm and the first 10 mm follicle. The purpose of this study was to compare LH, FSH, and Estradiol - 17β (E2) concentrations and to assess the follicular dynamics in control (C = non-hyperstimulated) versus treated (HS = hyperstimulated) mature, non-lactating Holstein cows (n = 4 / group) during the perideviation period. Controls received intramuscular (IM) saline injections and the HS group received 50 mg FSH injected IM 2x daily for 4 days beginning 48h after follicular ablation of all follicles > 4 mm and initiated prior to the perideviation period. Daily blood samples began at the initiation of an engineered follicular wave (48h post follicular ablation) and ended at ovulation. Additionally, jugular blood samples were collected at 15-min intervals during the perideviation period. To map follicular dynamics, ultrasonography was performed at 8h intervals prior to and 4h intervals during the perideviation period, and daily thereafter until ovulation. Serum samples were analyzed for LH, FSH, and E2 concentrations using specific RIAs. A

Student's t-test was used to compare the means of C and HS animals (mean ± standard error). The length of the perideviation period was 27.8h ± 2.1h in the C and 15.4h ± 1.6h in the HS group (P = 0.003). The number of 8 mm follicles at the beginning of the perideviation period was 2.3 ± 0.3 in the C and 6.0 ± 1.8 in the HS group (P = 0.082). The number of ovulations were 1.0 ± 0.0 in the C and 7.5 ± 3.2 in the HS group (P = 0.087). The average FSH concentration (ng / ml) during the perideviation period was 0.8 ± 0.1 in the C and 1.1 ± 0.1 in the HS group (P = 0.066). The E2 concentration (pg / ml) from ablation to ovulation was 10.2 ± 1.3 in the C and 17.2 ± 0.8 in the HS group (P = 0.003). At the beginning of the perideviation period, the E2 concentrations were 8.9 ± 2.5 in the C and 18.9 ± 1.0 in the HS group (P = 0.01). The E2 concentration per preovulatory follicle was 10.2 ± 1.3 in the C and 3.2 ± 0.7 in the HS group (P = 0.003). The LH values were ordered (highest to lowest) for each animal and the boundary for the top 25 percent of values was determined. A pulse was defined as two consecutive points ≥ the top 25 percentile. The LH pulses were 6.8 ± 0.9 in the C and 3.3 ± 0.3 in the HS group (P = 0.008). These results indicated group differences in the length of the perideviation period, E2 concentrations from ablation to ovulation, at the start of perideviation, and per preovulatory follicle, and LH pulsitility. These data further indicate a trend in the number of 8 mm follicles at the beginning of the perideviation period, the number of ovulations, and FSH concentrations. The increase in E2 concentration prior to morphological selection is of interest because it underscores the possibility that physiological dominance may precede morphological dominance. Future research is necessary to evaluate the influence of the number of dominant follicles on endocrine

profiles and to determine the timing of increased E2 production relative to morphological dominant follicle establishment.

Appendix E

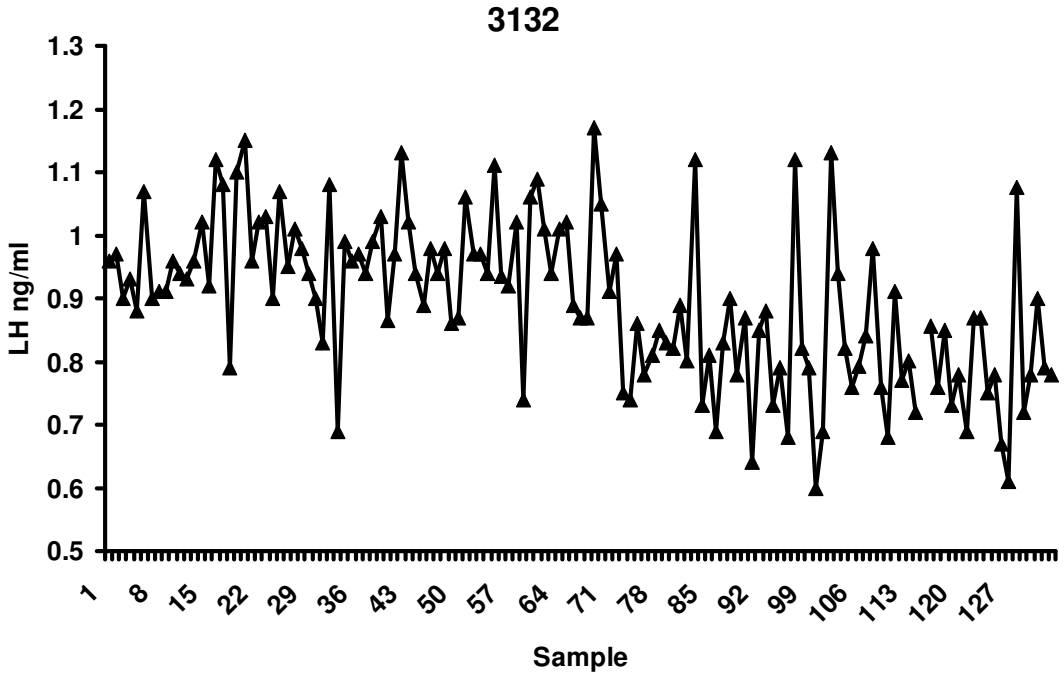
Society for the Study of Reproduction Poster 2007

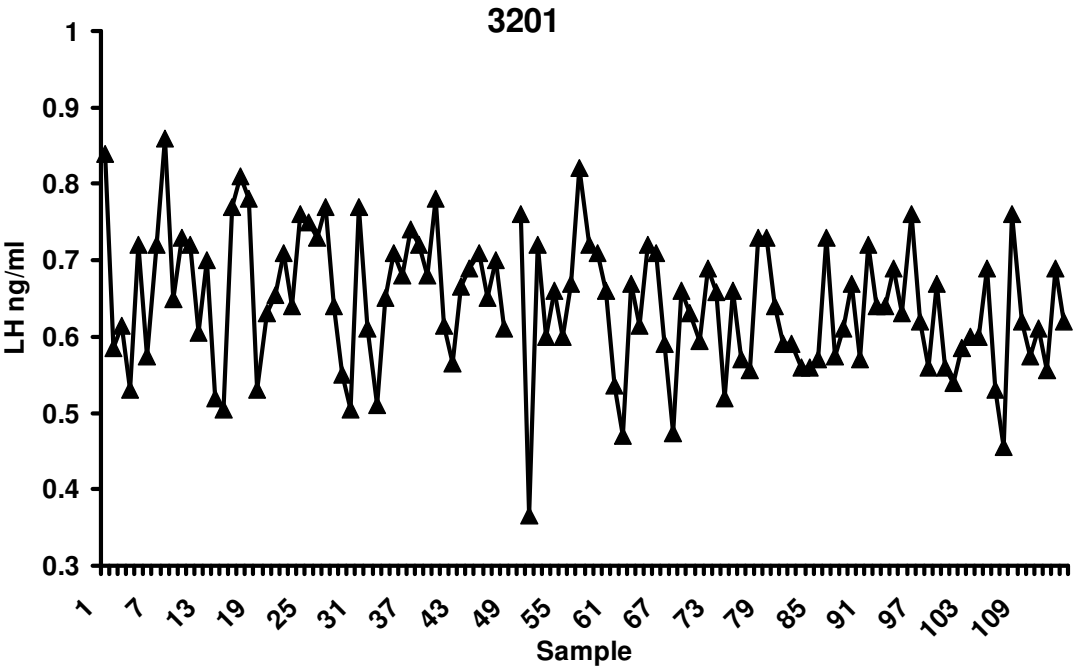


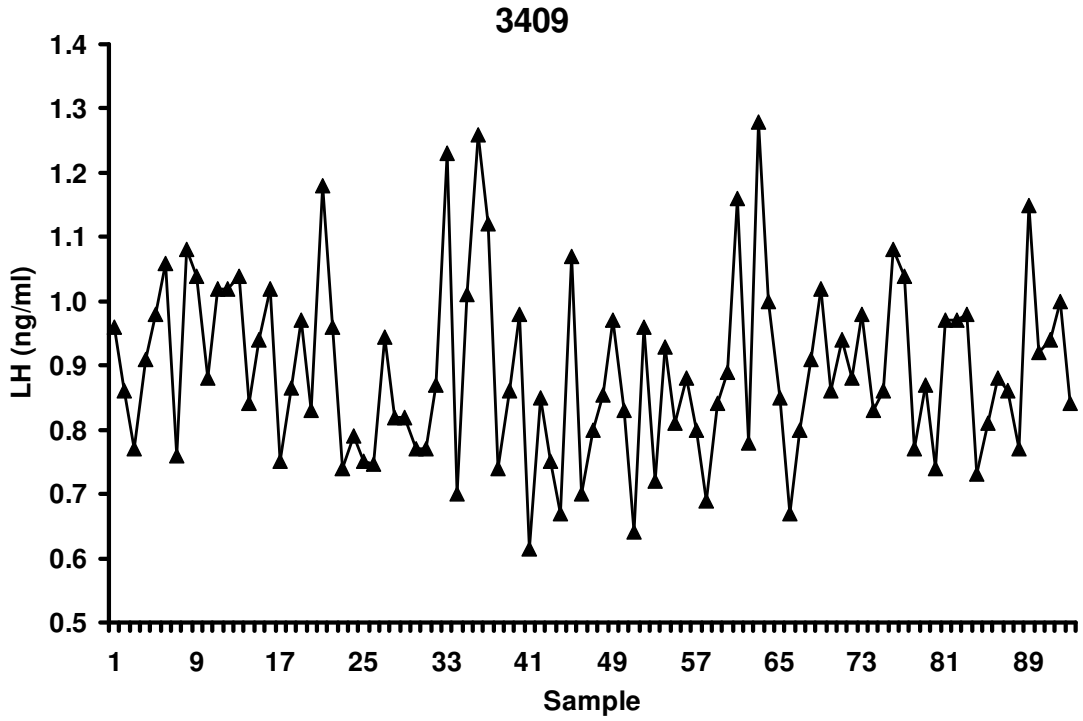
Appendix F

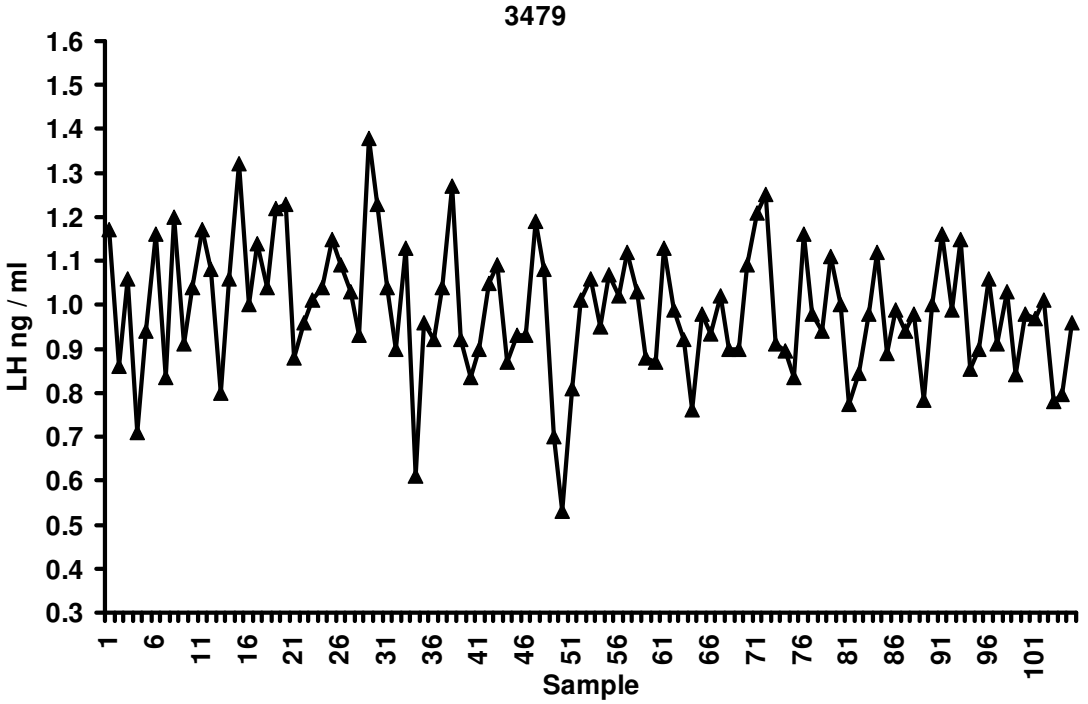
Individual Cow LH Data for Experiment 1

Experiment 1 - Control Cows – Individual LH Data (ng / ml)

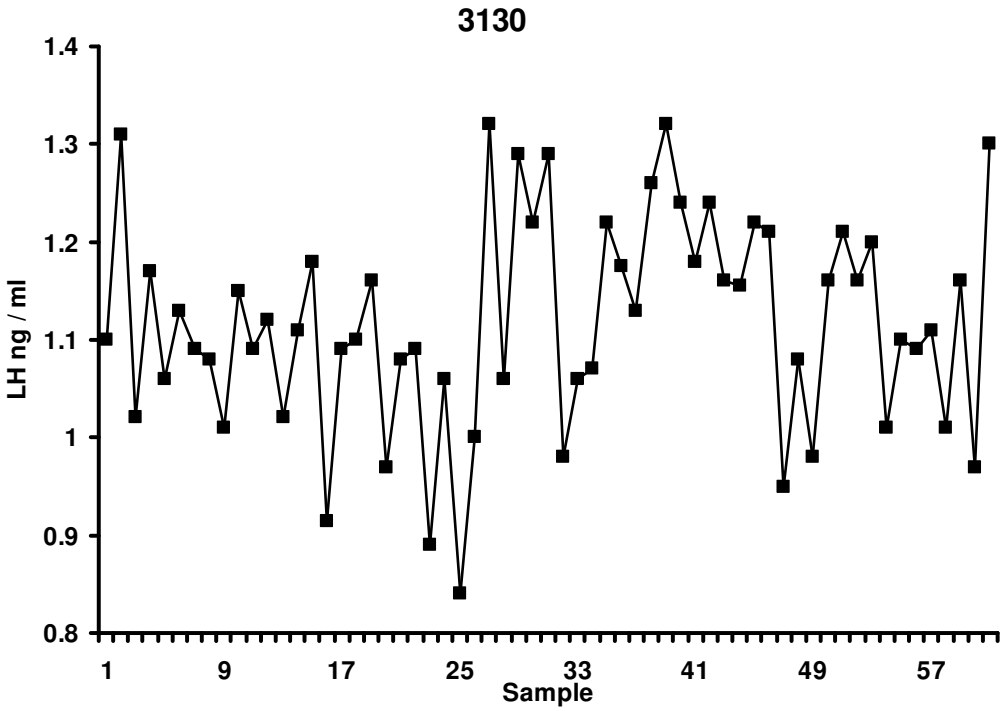


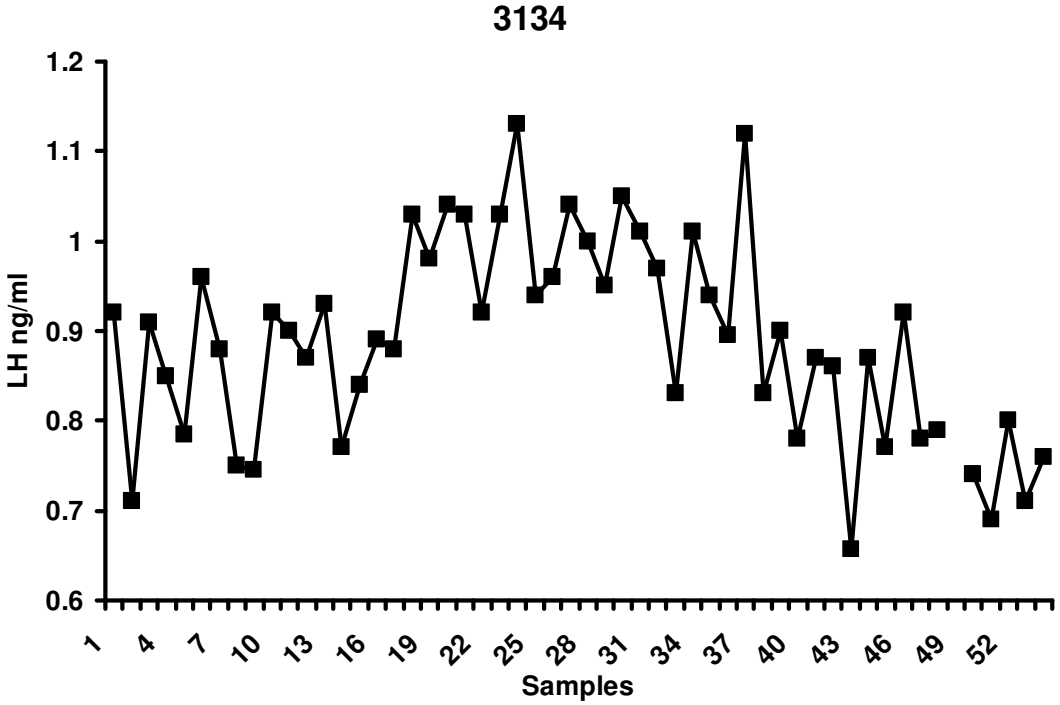


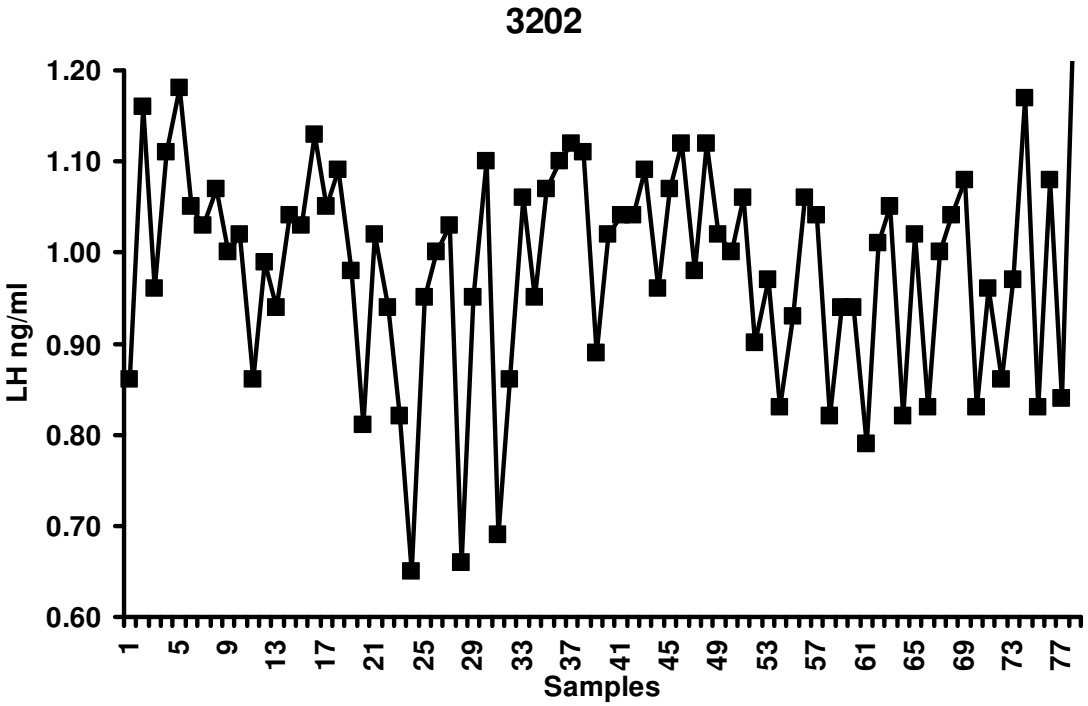

3409
LH (ng/ml)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
1
9
17
25
33
41
49
57
65
73
81
89
Sample

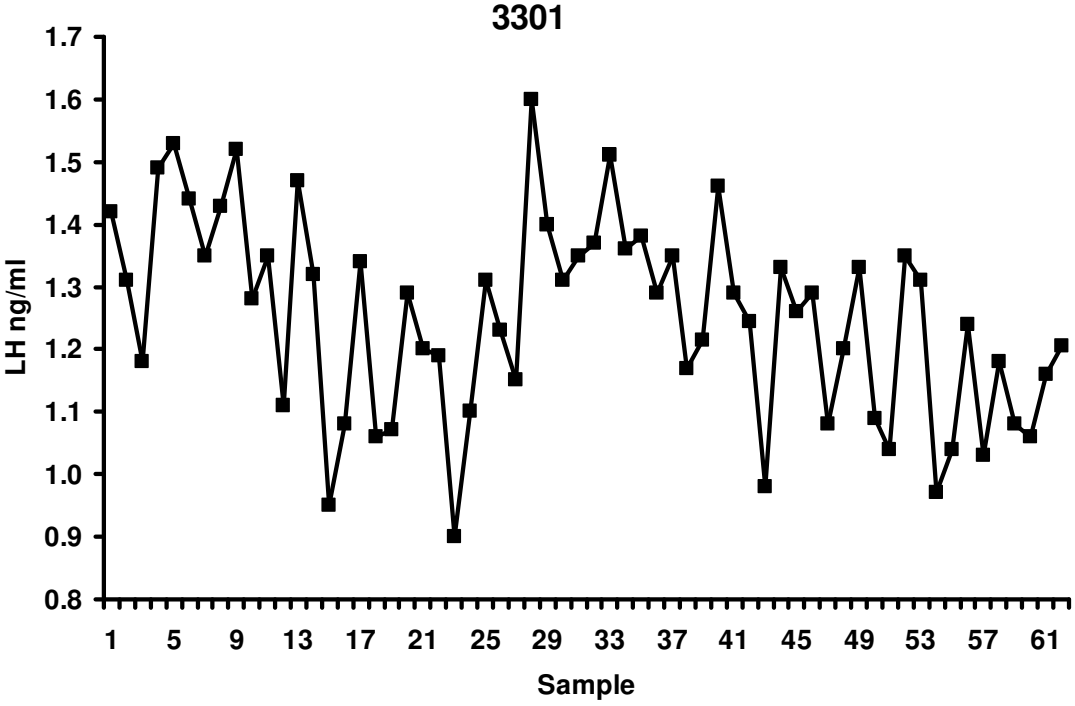

3479
LH ng / ml
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
1
6
11
16
21
26
31
36
41
46
51
56
61
66
71
76
81
86
91
96
101
Sample

Experiment 1 - Hyperstimulated Cows – Individual LH Data (ng / ml)






3202
LH ng/ml
1.20
1.10
1.00
0.90
0.80
0.70
0.60
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77
Samples


3301
LH ng/ml
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61
Sample

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